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13. ABSTRACT ***(Maximum 200 Words)***

Cyclin D1 and cyclin E are overexpressed in approximately 45% and 30% of breast cancer and overexpression of these two oncogenes is associated with poor prognosis. To define the role of cyclin D1 and cyclin E expression as markers of therapeutic responsiveness, cell lines overexpressing cyclin D1 or cyclin E were produced. Constitutive overexpression of cyclin D1 in the range of 5 fold and cyclin E in the range of 3-6 fold were confirmed by Western blots. Measurement of S-phase fraction up to 72 hours and colony-forming assay up to 3 weeks of treatment with progestin and antiestrogen were assessed. Overexpression of cyclin D1 but not cyclin E induced progestin resistance in both short- and long-term treatment. Overexpression of cyclin D1 decreased sensitivity to antiestrogen inhibition at 24 and 48 hours, while overexpression of cyclin E was less pronounced. Antiestrogen treatment inhibited colony growth in cyclin D1- or cyclin E-overexpressing cells following 3 weeks of treatment, but with a 2-2.5 fold decrease in sensitivity. Sensitivity to long-term antiestrogen was associated with downregulation of cyclin D1 protein levels. Colony-forming assay failed to demonstrate any effect of cyclin D1 or cyclin E overexpression on sensitivity to a range of chemotherapeutic agents including doxorubicin, methotrexate, 5-fluorouracil, paclitaxel. Ongoing translational research to assess the relationship between progestin/antiestrogen sensitivity and expression of cyclin D1 or p27 in the clinical setting will provide more insight into the usefulness of cyclin D1 in selecting the most efficacious endocrine therapy and its contribution to endocrine resistance in ER-positive breast cancer.

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Introduction

Cyclins belonging to the D and E families and their respective kinase partners play a pivotal role in regulating the progression of diverse cell types through G_1 phase of the cell cycle. Deregulated expression of either cyclin D1 or cyclin E can provide a growth advantage to tumor cells; their expression in the mammary gland of transgenic mice results in abnormal epithelial proliferation and adenocarcinoma, and they thus function as oncogenes. Cyclin D1 and cyclin E are overexpressed in approximately 45% and 30% of human breast cancer. Overexpression of these 2 genes is associated with poor prognosis in primary breast cancers. The latter could be due in part to reduced responsiveness of the tumors to systemic treatment. *In vitro* studies from our laboratory indicated that ectopic overexpression of cyclin D1 in breast cancer cells abrogated the growth-inhibitory effects of antiestrogens, suggesting indirectly that overexpression of cyclin D1 may confer resistance to endocrine treatment. The aim of this project was to define the role of cyclin D1 and cyclin E as markers of therapeutic responsiveness in both preclinical and clinical models of breast cancer. In the short term, this may aid in breast cancer management by potentially providing a better choice of treatment for each individual breast cancer patient. In the long-term, understanding the underlying molecular mechanisms contributing to therapeutic responsiveness in cyclin D1 / cyclin E overexpressing tumors may identify potential targets for modulation of drug resistance in breast cancer therapy.

The project was designed to:

1. assess the relationship between cyclin D1/cyclin E expression and response to therapy including endocrine treatment and chemotherapy in *in vitro* studies.
2. determine the underlying mechanisms contributing to cyclin D1 and cyclin E altering response to breast cancer therapies *in vitro*.
3. investigate the relationship between the levels of cyclin D1 and cyclin E expression, response rate and survival in the clinical setting.

Body of Report

Task 1: *In vitro* study to determine the relationship between cyclin D1/cyclin E expression and response to cancer therapy

Breast cancer cell lines constitutively overexpressing cyclin D1 or cyclin E were produced and characterized. These cell lines were then used to address whether cyclin D1 and cyclin E were predictive markers for therapeutic responsiveness in breast cancer.

Both estrogen and progesterone receptors are used routinely as predictive markers of responsiveness to hormonal therapy in breast cancer and they have been used for years as therapeutic targets. Estrogens and progestins exert their effects in the G_1 phase of the cell cycle and cyclin D1 or cyclin E are down-stream targets through which antiestrogens or progestins mediate their anti-proliferative effect. Previous *in vitro* studies from our laboratory clearly demonstrated that ectopic induction of cyclin D1 expression in ER-positive breast cancer cell

lines (T-47D and MCF-7) can overcome the inhibition of cell cycle progression induced by antiestrogen (1) suggesting indirectly that cyclin D1 overexpression may confer resistance to endocrine treatment. However, another study showed that tetracycline-regulated cyclin D1 overexpression in MCF-7 breast cancer cells does not prevent inhibition of cell growth by antiestrogens (2). A clinical study from this laboratory suggested that the duration of the response to tamoxifen was significantly longer in ER-positive patients with low cyclin D1 mRNA levels than in those with high cyclin D1 (3), implying that overexpression of cyclin D1 may confer a degree of resistance to antiestrogen therapy, although the sample size in the subgroup treated with antiestrogen in this study was small and the analyses must therefore be interpreted with caution. We, therefore, in the first instance, tested whether overexpression of cyclin D1 or cyclin E resulted in resistance to antiestrogens and progestins in the ER-positive T-47D human breast cancer cell lines. The effects of the pure steroidal antiestrogen, ICI 182780, and the progestin, ORG 2058, on cell proliferation in cell lines constitutively overexpressing cyclin D1 (by 5 fold) or cyclin E (by 3 - 6 fold) were investigated using flow cytometry for S-phase fraction and colony-forming assay for long term growth effects. The data are presented in full in the attached manuscripts (Appendices 1 and 2), and are summarized below.

In the parent T-47D cells and the empty vector cells, cyclin D1 gene expression is downregulated by antiestrogen and both cyclin D1 and cyclin E levels are reduced by progestin (Appendix 1 Fig 5, Appendix 2 Fig 1). In the cyclin D1 overexpressing cell line, cyclin D1 expression was maintained for at least 72 hours following treatment with ICI 182780 and ORG 2058. Similarly, cyclin E expression was not decreased up to 72 hours after ORG 2058 treatment of cyclin E overexpressing cell lines. Cyclin E levels were slightly reduced by ICI 182780 in the empty vector cells, but increased slightly 24 hrs following ICI 182780 treatment in both cyclin E overexpressing cell lines.

Treatment of the parent cell line with ORG 2058 resulted in a marked reduction in S-phase fraction, while treatment with ICI 182780 led to a moderate reduction in S-phase at 48 hours. Treatment of cells overexpressing cyclin D1 with ORG 2058 only resulted in a slight reduction in S-phase at 48 hours indicating marked resistance to progestin inhibition (Appendix 1 Fig 3&4). Overexpression of cyclin E produced a similar effect to cyclin D1 overexpression, but to a lesser extent, indicating partial resistance to progestin (Appendix 1 Fig 4). Treatment of cyclin D1 overexpressing cell lines with ICI 182780 led to significant resistance to antiestrogenic effects on cell cycle progression at 24 hrs (Appendix 2 Fig 2). The cyclin E overexpressing cell lines demonstrated a much less significant effect at 24 hrs. By 48 hrs, D1 overexpressing cells became more sensitive to ICI 187280 and by 72 hrs, neither cyclin-overexpressing cell line showed any alteration in sensitivity compared to the control cell line (Appendix 2 Fig 2).

Long-term effects of progestin and antiestrogen on cell growth were investigated using a colony-forming assay. The cells were treated with ICI 182780 and ORG 2058 over a range of concentrations for 3 weeks. There was a marked reduction in sensitivity to progestin treatment in the cyclin D1 overexpressing cells, with a significant number of colonies apparent at 3 weeks in cyclin D1 overexpressing but not control cell lines (Appendix 1 Fig 1). Overexpression of cyclin

E conferred partial resistance to growth inhibition by progestin (Appendix 1 Fig 1). In contrast, there was only a very slight decrease in the final level of growth inhibition induced by ICI 182780 in cyclin D1- or cyclin E-overexpressing cells in the long-term colony-forming assays (Appendix 2 Fig 6). They did, however, require a concentration of ICI 182780 ~2.5 times greater for the same degree of inhibition.

Colony-forming assays were also used to test responsiveness to a range of chemotherapeutic agents including doxorubicin, methotrexate, 5-fluorouracil and paclitaxel. These were tested over a wide (10^{-5} to 10^{-11}M) concentration range. The data suggest that neither cyclin D1 nor cyclin E overexpression exerted any significant effect on the sensitivity of the breast cancer cell lines to chemotherapy and this line of experimentation was therefore not pursued further.

Task 2: Study of the underlying mechanisms in determining sensitivity to cancer therapy

Several molecular endpoints have been identified following acute (0 - 48 hrs) treatment of MCF-7 breast cancer cells with the antiestrogen ICI 182780 in other studies from our laboratory. Inhibition of cyclin D1 gene expression with concurrent decline in cyclin D1 mRNA and protein levels is an early and critical event in antiestrogen action (4, 5). More recently, both cyclin D1-Cdk4 (5) and cyclin E-Cdk2 activity were shown to be inhibited by antiestrogen treatment and this decline was dependent on the CDK inhibitor p21 (6). Thus, experiments were performed to define the effects of cyclin D1 and cyclin E overexpression on key molecular endpoints, including phosphorylation of pRb, cyclin E-Cdk2 activity, p21 and p27 association with these complexes.

Following 24 hrs of treatment of the empty vector cells with ICI 182780, the total amount of pRb decreased and the hypophosphorylated form of pRb predominated. In contrast, hyperphosphorylated pRb remained abundant following treatment of the cyclin D1-overexpressing cell line D1 17-1 (Appendix 2, Fig 3), consistent with resistance to early cell cycle inhibition. The two cyclin E overexpressing cell lines displayed an intermediate effect on pRb phosphorylation suggesting partial resistance (Appendix 2, Fig 3). The cell line expressing higher levels of cyclin E showed a greater degree of resistance. These findings are consistent with the assessment of cell proliferation by S-phase fraction after 48 hours of ICI 182780 treatment (task 1). These results were also consistent with kinase activities of cyclin E-Cdk2 and cyclin D1-Cdk4. The abundance of Cdk4-phosphorylated pRb was maintained in the D1 17-1 cells, but reduced in the empty vector and E 17-2 cells after treatment with ICI 187280 (Appendix 2, Fig 3), indicating that cyclin D1-Cdk4 kinase activity is maintained by overexpression of cyclin D1. The cyclin E-Cdk2 activity was maintained slightly longer in the cyclin E overexpressing cells compared with the empty vector cells (Appendix 2, Fig 3). The cyclin E-Cdk2 activity was maintained in the cyclin D1-overexpressing cells for at least 24 hours following ICI 187280 treatment, consistent with continued cell proliferation as evidenced from data on pRb phosphorylation and S phase fraction

A substantial increase in the amount of cyclin E-Cdk2-associated p21 and p27 was demonstrated in MCF-7 breast cancer cells following treatment with antiestrogen (6). The association with the

CDK inhibitors may be altered in the presence of overexpression of cyclin D1 or cyclin E and any redistribution of CDK inhibitors may affect the kinase activity and in turn phosphorylation of pRb. Given that the p16 gene is silenced by hypermethylation of the gene promoter in T-47D cells (7), immunoblot analyses of the two predominant CDK inhibitors, p21 and p27 were performed in the cyclin D1 and cyclin E overexpressing cell lines (Appendix 2, Fig 4&5). The abundance of total p21 protein in empty vector cells peaked after 15 hrs of ICI 182780 treatment, while p27 levels increased at 15 hrs and were maintained until 48 hrs of ICI 182780 treatment. Following antiestrogen treatment of the cyclin D1 overexpressing cells, both p21 and p27 levels increased much earlier (by 6 hrs) and were maintained longer (until at least 48 hrs), suggesting lower protein turnover of p21 or p27 when they are bound in complexes. The abundance of both cyclin D1-p21 and cyclin E-p21 complexes was reduced by antiestrogen treatment in empty vector, control cells (Appendix 2 Fig 4). In contrast, both cyclin D1- and cyclin E-associated p21 levels were maintained in the cyclin D1-overexpressing cells. In the cyclin E-overexpressing cells following treatment with ICI 187280, although the abundance of cyclin D1-p21 complexes decreased, the abundance of cyclin E-p21 complexes increased. p27 levels increased modestly following treatment with ICI 187280 in all cell lines. Little change in cyclin D1-p27 association was apparent in any of the cell lines (Appendix 2 Fig 5). However, cyclin E-p27 association increased following ICI 187280 treatment in all the cell lines. The greater increase in p21 and p27 association with cyclin E-Cdk complexes by 48 to 72 hrs in E 17-2 cells may account for the more effective inhibition of cell proliferation by antiestrogen in the E 17-2 cells as compared to the D1 17-1 cells.

Western blots of lysates from cyclin D1 overexpressing cells treated with ICI 182780 for 7 and 10 days indicated that cyclin D1 protein levels are markedly reduced by antiestrogen treatment and there was also an accompanying reduction in cyclin E-Cdk2 kinase activity (Appendix 2 Fig 7). Half-life experiments currently in progress suggest that this is likely due to increased degradation of the cyclin D1 protein. The downregulation of cyclin D1 in the constitutively overexpressing cell line may thus account for the discrepancies between the short-term and long-term effect of antiestrogen treatment. We can therefore postulate that downregulation of cyclin D1 by antiestrogen plays a pivotal role in drug sensitivity. The development of resistance to tamoxifen is inevitable in the management of breast cancer patients. Our data suggest that one of the mechanisms contributing to antiestrogen resistance may be failure to downregulate cyclin D1. If so, cyclin D1 may be a potential target for modulation of resistance to antiestrogen in ER-positive breast cancers.

Although cyclin E levels were largely unchanged 7 - 10 days following ICI 187280 treatment of the cyclin E-overexpressing cells, the substantial increase in the cyclin E-p27 association was maintained at these late time points (Appendix 2 Fig 7), suggesting that this association played an important role in the inhibition of cell growth following long-term treatment with antiestrogen.

In a parallel study, the effect of cyclin D1 and cyclin E overexpression on progestin treatment was also studied (Appendix 1). Western blots indicated that both cyclin D1 and cyclin E expression were downregulated by 60% following treatment of empty vector cells with ORG

2058. In contrast, cyclin D1 expression was maintained in cyclin D1 overexpressing cells following ORG 2058 treatment, while cyclin E levels decreased after 24 hour in the cyclin D1 overexpressing cells but recovered to 80% of control by 48 hour (Appendix 2 Fig 5). In the cyclin E overexpressing cell lines, cyclin E expression was reduced little following progestin treatment, but cyclin D1 expression decreased to an extent similar to that in control cells (Appendix 1 Fig 5).

Cyclin E-Cdk2 activity was measured using an *in vitro* kinase assay. Cyclin E-Cdk2 activitiy decreased by only 60-65% following ORG 2058 treatment of cyclin E overexpressing cells compared with the 90% decrease in control cells (Appendix 1 Fig 6). Because cyclin E overexpressing cells displayed a 2-fold higher basal level of cyclin E-Cdk2 activity than empty vector cells, cyclin E-Cdk2 kinase activity was maintained at a level following progestin treatment similar to that in untreated control cells. Although the abundance of cyclin E protein was maintained following progestin treatment of the cyclin E overexpressing cells, increased cyclin E-p27 association likely accounted for the decrease in cyclin E-Cdk2 activity (Appendix 1 Fig 7). There was no change in p21 association with cyclin E following progestin treatment.

In cyclin D1 overexpressing cells, the cyclin E-Cdk2 kinase activity initially reduced to a similar magnitude to that in control cells, but subsequently recovered from 25% of control to 50% of control by 48 hours following progestin treatment (Appendix 1 Fig 8), in parallel with the increase in cyclin E expression at that point. The association between p27 and cyclin E was initially increased by ORG 2058 treatment and this association was maintained at 48 hour (Appendix 1 Fig 7), indicating that the partial recovery of the cyclin E-Cdk2 activity at 48 hour was not due to redistribution of p27. Overall these data indicate that cyclin D1 is a critical element of progestin inhibition of proliferation in breast cancer cells. Cyclin D1 overexpression leads to progestin resistance in breast cancer, but this is not dependent on sequestration of p27.

Task 3: Clinical studies to determine the relationship between cyclin D1/cyclin E expression and response to cancer therapy

Unfortunately, the sourcing of paraffin-embedded tissue blocks from patients with advanced breast cancer from ANZ breast cancer trials 7802 and 8101 treated with various chemotherapeutic and endocrine regimens has been delayed due to circumstances beyond our control. Other collaborations in Sydney and overseas were negotiated to test whether cyclin D1 and cyclin E overexpression are markers of therapeutic responsiveness in endocrine and radiation treatment in well-characterized cohorts of breast cancer patients. These studies are ongoing. Slides from 40 paraffin-embedded tissue blocks were available through collaboration with Professor Mitch Dowsett, Royal Marsden Hospital, London, UK. The patients were randomised to receive neoadjuvant hormonal therapy either tamoxifen or letrazole prior to surgery. The immunohistochemical staining of cyclin D1 and cyclin E expression in these primary breast cancers has been completed and is currently undergoing analysis. The relationship between response to tamoxifen or letrazole, as measured by a series of surrogate markers, and expression of cyclin D1 or cyclin E will be examined. This continuing collaboration using an established

paradigm (8) is expected to allow assessment of relationships between cyclins D1 and E, other newly defined markers of endocrine resistance, and patient response using a variety of endocrine therapies over the next few years.

Key Research Accomplishments

- Development of clonal ER-positive T-47D breast cancer cell lines constitutively overexpressing cyclin D1 or cyclin E.

- Demonstration that ectopic overexpression of cyclin D1 or cyclin E confers progestin-independent growth of breast cancer following both short and long term exposure. Overexpression of cyclin D1 effectively induces progestin resistance, while cyclin E overexpression does so to a lesser extent.

- Demonstration that overexpression of cyclin D1 or cyclin E interferes with the early cell cycle effects of antiestrogen treatment, but not long-term growth inhibition.

- Confirmation that downregulation of cyclin D1 by antiestrogen is a critical element determining sensitivity to antiestrogen and identification of a new mechanism for this response i.e. increased degradation of cyclin D1 protein after long-term antiestrogen but not progestin treatment.

- Progestin treatment of cyclin D1 overexpressing cells was accompanied associated with p27 association with cyclin E-Cdk2, demonstrating that the ability of cyclin D1 to confer progestin resistance does not depend on sequestration of p27.

- Ongoing translational studies to evaluate the relationship between biological markers of endocrine resistance, including cyclin D1 and cyclin E, and patient response to endocrine therapies.

Reportable Outcomes

Manuscripts

1. Cyclin D1 overexpression leads to progestin resistance in T-47D breast cancer cells without sequestration of $p27^{Kip1}$.
   Elizabeth A. Musgrove, Lisa-Jane Hunter, Christine S. L. Lee, Alexander Swarbrick, Rina Hui and Robert L. Sutherland.
   Journal of Biological Chemistry 276: 47675 – 47683, 2001. (Appendix 1)

2. Constitutive overexpression of cyclin D1 but not cyclin E confers acute resistance to antiestrogens in T-47D breast cancer cells.

Rina Hui, Georgina Finney, Christine S. L. Lee, Elizabeth A. Musgrove and Robert L. Sutherland.
Cancer Research, under revision. (Appendix 2)

Abstracts:

1. The Fourth Leura International Breast Cancer Conference, November 15 - 19th 2000.
   Constitutive overexpression of cyclin D1 or cyclin E prevents growth-inhibitory effects of progestin and antiestrogen
   Hui, R., Lee, C. S. L., Hunter, L. J., Finney, G., Musgrove, E. A., Sutherland, R. L.

2. The 13th Lorne Cancer Conference, February 8 - 11th 2001.
   Cell cycle control in breast cancer: mechanisms of CDK inactivation by progestins
   Musgrove, E.A., Swarbrick, A., Lee, C.S.L., Hunter, L.J.K., Hui, R. and Sutherland, R. L.

3. The 13th Lorne Cancer Conference, February 8 - 11th 2001.
   Role of cyclin E in progestin inhibition of proliferation
   Hunter, L.J.K., Lee, C.S.L., Hui, R., Sutherland, R.L. and Musgrove, E.A.

Presentations:

1. PI was invited to speak in the Basic Sciences of Oncology Series at the NSW Cancer Council on:
   - Molecular biology in breast cancer
   - Endocrine therapy in breast cancer

2. PI was invited to speak in the Oncology Meeting at Prince of Wales Hospital, Sydney on:
   - G_1 cyclins in breast cancer

Development of cell lines:

Clonal lines of T-47D cells stably transfected with empty pTRE vector and pTRE vector containing cyclin D1 and cyclin E have been established. 2 clonal lines overexpressing cyclin D1, 2 clonal lines overexpressing cyclin E and 1 vector-alone control clonal line were characterized.

Conclusions

The relationships between cyclin D1 or cyclin E expression and response to endocrine therapy (antiestrogen and progestin) and chemotherapy (doxorubicin, methotrexate, 5-flurouracil and paclitaxel) in the ER positive T-47D human breast cancer cell lines were examined using measurements of S-phase fraction and colony-forming assays. Our findings indicated that overexpression of cyclin D1 and to a lesser extent cyclin E confer resistance to progestin treatment. In contrast, overexpression of cyclin D1 or cyclin E appeared to interfere with the early cell cycle effects of antiestrogen, but the long-term antiestrogen-induced growth inhibition remained effective in both cyclin D1 or cyclin E overexpressing breast cancer cells. Downregulation of cyclin D1 levels by antiestrogen results in sensitivity of the cells to the

antiestrogen inhibition of cell proliferation in the long-term. The data suggest that cyclin D1 expression and cyclin E-p27 association are important in the underlying mechanisms of antiestrogen action. Cyclin D1 overexpression leads to progestin resistance without sequestration of p27, indicating that regulation of cyclin D1 is a critical element of progestin inhibition in breast cancer cells. Although resistance to progestin in cyclin D1-overexpressing breast cancers requires confirmation in the clinical setting, these *in-vitro* data suggest that breast cancers overexpressing cyclin D1 would respond poorly to progestin therapy. Further translational research to assess the relationship between tamoxifen sensitivity and level of expression of cyclin D1 or p27 in the clinical setting may provide more insight into the usefulness of cyclin D1 in selecting the most efficacious endocrine therapy and the contribution of cyclin D1 expression to the development of endocrine resistance in ER-positive breast cancer.

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Cyclin D1 Overexpression Induces Progestin Resistance in T-47D Breast Cancer Cells Despite $p27^{Kip1}$ Association with Cyclin E-Cdk2*

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Long-term growth inhibition, arrest in G_1 phase and reduced activity of both cyclin D1-Cdk4 and cyclin E-Cdk2 are elicited by progestin treatment of breast cancer cells in culture. Decreased cyclin expression, induction of $p18^{INK4c}$ and increased association of the CDK inhibitors $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ with cyclin E-Cdk2 have been implicated in these responses. To determine the role of decreased cyclin expression, T-47D human breast cancer cells constitutively expressing cyclin D1 or cyclin E were treated with the progestin ORG 2058. Overexpression of cyclin E had only a modest effect on growth inhibition. Although cyclin E expression was maintained during progestin treatment, cyclin E-Cdk2 activity decreased by ~60%. This was accompanied by $p27^{Kip1}$ association with cyclin E-Cdk2, indicating that both cyclin E down-regulation and $p27^{Kip1}$ recruitment contribute to the decrease in activity. In contrast, overexpression of cyclin D1 induced progestin resistance and cell proliferation continued despite decreased cyclin E-Cdk2 activity. Progestin treatment of cyclin D1-overexpressing cells was associated with increased $p27^{Kip1}$ association with cyclin E-Cdk2. Thus the ability of cyclin D1 to confer progestin resistance does not depend on sequestration of $p27^{Kip1}$ away from cyclin E-Cdk2, providing evidence for a critical function of cyclin D1 other than as a high-capacity "sink" for $p27^{Kip1}$. These data indicate that regulation of cyclin D1 is a critical element of progestin inhibition in breast cancer cells and suggest that breast cancers overexpressing cyclin D1 may respond poorly to progestin therapy.

The female sex steroid progesterone and its synthetic analogues, progestins, have complex effects on cell proliferation and can either stimulate or inhibit cell proliferation, depending on the cell type, tissue, or treatment regimen (1). For example, in the uterus progesterone acts synergistically with estrogen to stimulate stromal proliferation but inhibits estrogen-induced epithelial proliferation. The latter effect has led to the addition of progestin to hormone replacement therapies to counteract the increased risk of endometrial cancer arising from treatment with estrogen alone (2). Synthetic progestins have an established role in the therapy of breast and endometrial cancers (2, 3), demonstrating a growth-inhibitory effect on breast and endometrial cancer cells, although whether progesterone is stimulatory or inhibitory for normal breast epithelium remains controversial. Progestins have a biphasic effect on the proliferation of breast cancer cells in culture (4), initially stimulating G_1 cells to enter S phase, but the predominant effect is long term growth inhibition. Several recent studies have focused on the molecular mechanisms for this growth inhibition (5–7).

Progestin-mediated growth inhibition is preceded by decreased expression of the major G_1 cyclins in breast cancer cells, cyclin D1 and cyclin E (5, 6), and preferential formation of cyclin E-Cdk2 complexes that contain the CDK[1] inhibitor $p27^{Kip1}$ and are therefore inactive (6, 7). The related CDK inhibitor $p21^{Cip1}$ appears to play a minor role since immunodepletion experiments indicate that few of the cyclin D1- or cyclin E-containing complexes contain $p21^{Cip1}$ following progestin treatment (7). The increased association of $p27^{Kip1}$ with cyclin E-Cdk2 occurs prior to any increase in $p27^{Kip1}$ abundance. Since in breast cancer cells cyclin D-Cdk4 complexes bind a significant fraction of the total cellular $p27^{Kip1}$, a decrease in their abundance will make $p27^{Kip1}$ increasingly available to associate with other molecules and this likely contributes to increased $p27^{Kip1}$-cyclin E-Cdk2 association. The increased formation of $p27^{Kip1}$-cyclin E-Cdk2 complexes also reflects increased expression of another CDK inhibitor, $p18^{INK4c}$ (7). In contrast with $p27^{Kip1}$ and $p21^{Cip1}$, which associate with both cyclin D-Cdk4 and cyclin E-Cdk2, $p18^{INK4c}$ specifically inhibits the activity of cyclin D-associated kinases, restricting cyclin D binding to Cdk4/6 and thereby making $p27^{Kip1}$ and $p21^{Cip1}$ available to bind other proteins (8). Thus, it is apparent that progestins target multiple elements of the cell cycle control machinery which may contribute to inhibition of CDK activity and consequent inhibition of proliferation. However, the relative importance of regulating cyclin abundance remains unclear, and it was the objective of this study to address this issue by using constitutive overexpression of cyclin D1 or cyclin E as a means of maintaining cyclin expression during progestin treatment.

Additional impetus for these experiments comes from the frequent overexpression of cyclin D1 or cyclin E in breast cancer. Cyclin D1 is overexpressed in ~50% of breast cancers (9–11). The consequences of this for patient prognosis are not clear, with conflicting data from early studies (12–16). More recent data indicate that cyclin D1 overexpression is an indicator of poor prognosis specifically in estrogen receptor (ER)-

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§ Recipient of an Australian Postgraduate Award.

[1] The abbreviations used are: CDK, cyclin-dependent kinase; ER, estrogen receptor; GFP, green fluorescent protein; GST, glutathione *S*-transferase; pRb, retinoblastoma protein.

positive breast cancers (17). Cyclin E is present as low molecular weight isoforms in breast cancer but not in normal breast epithelium and is overexpressed in ~30% of breast cancer specimens (18–21). This is associated with significant increases in the risk of death or relapse (18, 21). One possible explanation for poor outcome associated with cyclin overexpression is impaired response to therapies that modulate cyclin D1 expression, but there are to date few studies addressing the question of whether cyclin overexpression might alter response.

This manuscript demonstrates that overexpression of cyclin D1 induces resistance to progestin-mediated growth inhibition, but overexpression of cyclin E has little effect. Maintenance of cyclin D1 expression after progestin treatment did not prevent increased $p27^{Kip1}$ association with cyclin E-Cdk2. This observation is inconsistent with the suggestion, supported by recent genetic evidence (22, 23), that sequestration of $p27^{Kip1}$ is the physiologically relevant function of cyclin D1. Rather, it indicates that in the context of progestin inhibition, other functions of cyclin D1 are critical. Overall, the data presented here indicate that regulation of cyclin D1 is a central element of progestin inhibition of breast cancer cell proliferation and suggest that breast cancers overexpressing cyclin D1 may respond poorly to progestin therapy.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture—The generation of T-47D human breast cancer cells constitutively expressing either cyclin D1 or cyclin E is described fully elsewhere.[2] In brief, a T-47D clone stably transfected with the tet-responsive transcriptional activator tTA (T-47D tTA-17) was transfected with a pTRE vector containing full-length cyclin D1 or full-length cyclin E cDNA. Stable clones were selected by hygromycin treatment (200 μg/ml) following co-transfection with pTK-Hyg to produce cell lines overexpressing cyclin D1 (D1 17-1) or cyclin E (E 17-2, E 17-3). Western blot analysis of cell lysates demonstrated overexpression of the cyclin proteins in the absence of tetracycline. T-47D tTA-17 and clonal derivatives stably transfected with the empty pTRE vector were used as control cell lines. T-47D tTA-17 derivatives were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, insulin (10 μg/ml), and tetracycline (2 μg/ml).

T-47D-*Eco*R-p cells for retroviral infection were generated by stable transfection of a vector encoding the murine (ecotropic) retroviral receptor (pBabePuro-*Eco*R; provided by Dr. Gordon Peters, Imperial Cancer Research Fund, London, UK) into T-47D breast cancer cells. A clone was selected based on high retroviral infectability and normal steroid responsiveness and expanded for subsequent experiments.

RPMI 1640 medium supplemented with 5% fetal calf serum, insulin (10 μg/ml), and gentamicin (20 μg/ml) was used to culture the cell lines for progestin treatment experiments. The synthetic progestin ORG 2058 (16α-ethoxy-21-hydroxy-19-norpregn-4-en-3,20-dione; Amersham Biosciences) was dissolved in ethanol at 1,000-fold final concentration and added to cells in exponential growth. Control cultures received ethanol vehicle to the same final concentration.

Colony-forming Assay—The sensitivity of the cell lines to ORG 2058 treatment was assessed in colony-forming assays. Cells (8000 cells/plate) were plated into duplicate 6-cm diameter dishes in RPMI 1640,5% fetal calf serum. After 24 h, the cells were treated with a range of ORG 2058 concentrations (0.01–100 nM) or ethanol vehicle (control) and incubated for up to 35 days (typically 18–21 days) until the control dishes for each cell line reached similar colony size. The medium was changed, and ethanol or ORG 2058 treatment repeated every 7 days during this time period. The cells were fixed and stained using the Diff-Quik Stain Set (64851, Lab Aids Pty. Ltd., Narrabeen, Australia). The number of colonies in each dish was quantitated using Bio-Rad Quantity One 4.2.1 GelDoc software (Bio-Rad Laboratories, Hercules, CA).

Retroviral Infection—Retroviral vectors were constructed as follows: pLib-D1 was made by digesting pLib (CLONTECH Laboratories, Palo Alto, CA) with *Eco*RI/*Not*I and pHsCYCD1-H124 (David Beach, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) with *Eco*RI/*Hin*dIII, followed by agarose gel electrophoresis purification. The fragments were end-blunted with DNA polymerase and ligated. The pLib-cycE vector was constructed similarly, using a cyclin E long-form insert isolated from pBSSK-cycE (Steven Reed, Scripps Research Institute, La Jolla, CA) by *Eco*RI/*Hin*dIII digestion.

Ecotropic retroviruses expressing cyclins D1 and E were packaged in Phoenix-Eco cells (a gift of Philip Achacoso and Garry Nolan, Stanford University Medical Center, Stanford, CA) by transient transfection. Phoenix-Eco cells were seeded into 10-cm diameter dishes and 24 h later were transfected with 20 μg of vector DNA using 60 μl of FuGENE-6 reagent (Roche Diagnostics Australia, Castle Hill, New South Wales, Australia) per dish. The medium was changed 24 h later, and the cells incubated at 32 °C for a further 24 h. Viral supernatant was harvested by filtration (Millex-HV Durapore, Millipore Bedford, MA), and polybrene (Sigma) added to a final concentration of 16 μg/ml. Viral supernatant was added immediately to target cells by 1:4 dilution in culture medium or stored at −80 °C for later use. As a means of estimating infection efficiency a GFP-expressing virus (pLib-EGFP, CLONTECH) was packaged in parallel with the cyclin constructs.

T-47D-*Eco*R-p cells (5×10^5 cells/plate) were plated into 10-cm diameter dishes in RPMI 1640,5% fetal calf serum. After 24 h, the cells were infected with either the GFP, cyclin D1, or cyclin E viral supernatant and incubated with occasional swirling. The medium was removed 24 h later, and the cells were reinfected with fresh viral supernatant to maximize infection efficiency. Following a further 24 h of incubation, the retroviral-infected cells were plated (8000 cells/plate) into replicate 6-cm diameter dishes in RPMI 1640,5% fetal calf serum, subsequently treated with ORG 2058 or ethanol vehicle, stained after 18 days incubation, and quantitated as described above for the colony-forming assay. Parallel dishes were harvested for analysis of cyclin expression levels by Western blotting. GFP-expressing cells were also harvested for flow cytometric analysis 48 h after infection to estimate the proportion of infected cells, which was typically 30–50%.

Cell Lysis—Cells were lysed as follows: cell monolayers were washed once with ice-cold phosphate-buffered saline and then scraped into ice-cold lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 200 μM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM NaF, and 20 μM MG132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; Z-Leu-Leu-Leu-CHO; Calbiochem-Novabiochem Corporation, Darmstadt, Germany)). At selected timepoints an aliquot of this suspension was diluted in RPMI 1640,5% fetal calf serum and stained for later flow cytometric DNA analysis by addition of ethidium bromide (50 μg/ml) and Triton X-100 (0.2%). The remainder was incubated for 5 min on ice, and the cellular debris was cleared by centrifugation (15,000 × *g*, 5 min, 4 °C). For Cdk4 assays kinase lysis buffer was used: 50 mM HEPES, pH 7.5, 1 mM dithiothreitol, 150 mM NaCl, 10% (v/v) glycerol, 0.1% Tween 20, 1 mM EDTA, 2.5 mM EGTA, 10 mM β-glycerophosphate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1.0 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium orthovanadate, 1 mM NaF, 20 μM MG132. The cells were lysed, and the lysates cleared as previously described (24). The cleared lysates were stored at −80 °C.

Western Blot Analysis and Immunoprecipitation—Cell lysates (600 μg) were immunoprecipitated by incubation (40 min, 4 °C) with $p27^{Kip1}$ (C-19-G) antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) followed by incubation (1 h, 4 °C) with protein G-Sepharose beads (Zymed Laboratories Inc., San Francisco, CA). Alternatively, lysates were additionally incubated with $p21^{Cip1}$ (C-19) antibody from Santa Cruz Biotechnology that had been previously chemically cross-linked to protein A-Sepharose beads (Zymed Laboratories Inc.) by incubation in dimethyl pimelimidate (5 mg/ml),sodium tetraborate (0.2 M, pH 9.0), for 30 min at room temperature, essentially as described previously (25). A control with no antibody (mock immunoprecipitation) was included for each sample. Following three rounds of immunoprecipitation, the combined immunoprecipitated proteins were washed with lysis buffer and eluted by the addition of glycine (0.1 M, pH 2.7) for 30 min at room temperature. The beads were cleared by centrifugation and the eluted proteins neutralized with the addition of Tris-HCl (1 M, pH 9.0) before addition of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (63 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% SDS, 5% β-mercaptoethanol). Following the addition of ferritin carrier protein, the immunodepleted supernatant was acetone-precipitated overnight at −80 °C and then resuspended in SDS-PAGE sample buffer.

Samples of immunoprecipitated or total protein (50–80 μg) in SDS-PAGE sample buffer were heated to 95 °C for 3 min, then separated by SDS-PAGE, and transferred to nitrocellulose. The membranes were

[2] R. Hui, G. L. Finney, C. S. L. Lee, E. A. Musgrove, R. L Sutherland, manuscript in preparation.

incubated (2 h at room temperature or overnight at 4 °C) with the following primary antibodies: cyclin E (HE12) antibody from Santa Cruz Biotechnology; cyclin D1 (DCS6) antibody from Novocastra Laboratories, Newcastle-upon-Tyne, United Kingdom; $p27^{Kip1}$ (K25020) antibodies from Transduction Laboratories, Lexington, KY.; pRb (14001A) antibody from PharMingen, San Diego, CA; and phospho-Rb (Ser-780) antibody from New England Biolabs Inc., Beverley, MA. Following incubation (1 h at room temperature) with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (Santa Cruz Biotechnology), specific proteins were visualized by chemiluminescence (PerkinElmer Life Sciences). Where the proteins of interest were of sufficiently different mobilities, membranes were incubated either sequentially or simultaneously with several primary antibodies.

Kinase Assays—The histone H1 kinase activity of cyclin E immunoprecipitates from 100 to 250 μg of cellular protein was measured as previously described (24), using 10 μg of histone H1 as substrate. The degree of background histone H1 phosphorylation, estimated from parallel control samples immunoprecipitated using beads without antibody, was typically near the limit of detection. Kinase activity of Cdk4 immunoprecipitates from 500 μg of cellular protein was measured using 10 μg of GST-$pRb^{773-928}$ fusion protein substrate as previously described (24). The degree of background phosphorylation in pRb phosphorylation assays was estimated from parallel control samples immunoprecipitated following blocking of specific antibody binding with the appropriate antigenic peptide. Following termination of kinase reactions, samples were incubated at 95 °C for 3 min in SDS-PAGE sample buffer and separated by SDS-12% PAGE.

Image and Data Analysis—Images captured by PhosphorImager (Molecular Dynamics 445 SI; Molecular Dynamics, Sunnyvale, CA) or, for chemiluminescence, by densitometer scanning (Molecular Dynamics PDSI) of x-ray film, were quantitated using IP Lab Gel H analysis software (Signal Analytics, Vienna, VA). Quantitation of protein levels by this method was linear over the range of intensities measured. All figures were compiled using Deneba Canvas 5.0 software.

Flow Cytometry—Flow cytometric analysis was performed on a FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) using CELLQuest 2.0 (Becton Dickinson Immunocytometry Systems) software. The proportion of cells in the G_1, S, and G_2/M phases of the cell cycle were calculated from the resulting DNA histograms using ModFit LT analysis software (Verity Software House, Inc., Topsham, ME).

RESULTS

Overexpression of Cyclin D1 but Not Cyclin E Induces Progestin Resistance—To determine the effect of cyclin overexpression on sensitivity to growth inhibition by progestins we used clonal derivatives of T-47D breast cancer cells that had been transfected with either cyclin D1 or cyclin E. The levels of cyclin expression achieved were ~5-fold greater than in parental or vector-transfected control cells for the cyclin D1-overexpressing line and >3-fold for the cyclin E overexpressing lines (Fig. 1*A*). This level of cyclin overexpression had no effect on the expression of other G_1 cyclins, nor on the levels of the CDK inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ (Fig. 1*A* and data not shown).

Colony formation over 3 weeks of monolayer culture was used to test the sensitivity of the cyclin-overexpressing clonal cell lines to long term inhibition of proliferation following progestin treatment. Vector-transfected cells were profoundly inhibited by the presence of the progestin ORG 2058. Very few colonies were apparent after treatment with ≥1 nM ORG 2058, representing >99% inhibition of colony formation (Fig. 1, *B* and *C*). The concentration-dependence of the inhibition of proliferation was similar to that previously observed in the parental T-47D cells using different methodology (26). In marked contrast, the cyclin D1-overexpressing cell line (cyclin D1 17–1) was poorly inhibited by ORG 2058 treatment even at the highest concentration used, 100 nM (Fig. 1, *B* and *C*). In two cyclin E-overexpressing cell lines (cyclin E 17–2 and 17–3) ORG 2058 clearly inhibited colony formation, to ~25% of control at 1–100 nM, although significant numbers of colonies were still apparent (Fig. 1, *B and C*).

These data suggested that cyclin D1 overexpression attenuated the antiproliferative effects of progestins, but cyclin E

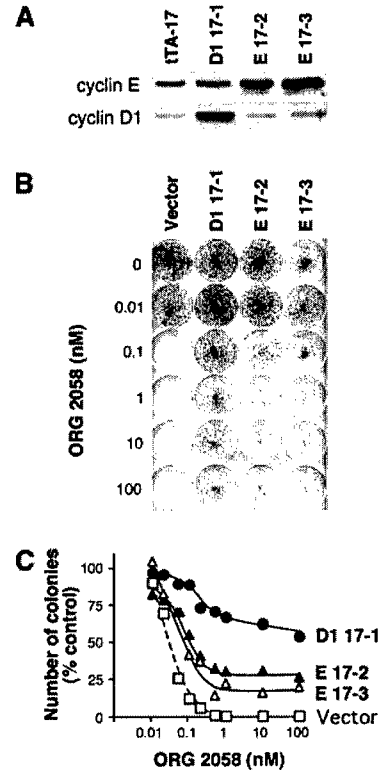

FIG. 1. **Progestin inhibition of proliferation in cells overexpressing cyclin D1 or cyclin E.** *A*, lysates of T-47D cells constitutively expressing cyclin D1 (D1 17–1) or cyclin E (E 17–2, E 17–3) were Western blotted for cyclins D1 and E in parallel with parental cells (tTA-17). *B* and *C*, T-47D cells constitutively expressing cyclin D1 (D1 17–1) or cyclin E (E 17–2, E 17–3) or vector-transfected control cells were treated with the indicated concentrations of progestin (ORG 2058) or vehicle. Cells were allowed to proliferate for 18–21 days before fixation, staining, and quantitation. Data are representative of two separate experiments. Graphical data are presented relative to the number of colonies in vehicle-treated cultures of each cell line.

overexpression had a more modest effect, still allowing substantial inhibition of proliferation. Measurement of estrogen and progesterone receptor levels by Western blot confirmed similar receptor expression in all the cell lines and demonstrated progestin-mediated down-regulation of both receptors in all cell lines (not shown), indicating that other progestin responses remained intact and thus that the apparent resistance was not simply due to defects in progesterone receptor expression or signaling.

To further confirm that the alteration in progestin sensitivity documented in Fig. 1 was due to cyclin overexpression rather than clonal variation, we infected T-47D cells with retroviruses expressing either GFP (control), cyclin D1, or cyclin E. Up to 50% of the original population was infected with virus. The resulting level of cyclin expression was similar to that in the cyclin-overexpressing clonal T-47D cell lines (Fig. 2) and was maintained over the 3 weeks of the experiment (not shown). Colony formation of cells infected with GFP-expressing virus was inhibited by ORG 2058 in a manner similar to other control T-47D derivatives, but retroviral expression of cyclin D1 resulted in marked resistance to ORG 2058-mediated growth inhibition (Fig. 2). Retroviral expression of cyclin E still allowed significant inhibition with the number of colonies reduced by ~60%, but again more colonies were apparent than in progestin-treated control cells. These experiments confirmed the conclusion obtained with the clonal cell lines overexpressing the cyclins *i.e.* that cyclin D1 overexpression markedly reduced sensitivity to progestin-mediated inhibition of proliferation, but cyclin E overexpression was much less effective.

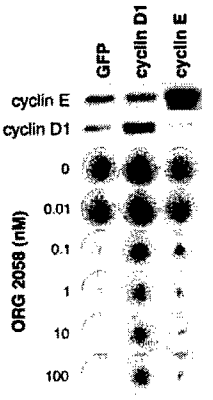

FIG. 2. **Effect of retroviral expression of cyclin D1 or E on progestin inhibition of proliferation.** T-47D cells expressing the ecotrophic retroviral receptor were infected with retroviruses expressing either GFP, cyclin D1, or cyclin E. *Upper panel* shows a Western blot of cyclin expression 48 h after infection. The infected cells were replated into replicate dishes and treated with the indicated concentrations of ORG 2058 or vehicle. After 18 days the cells were fixed and stained (*lower panel*). Data are representative of two separate experiments.

Acute Effects of Progestin Treatment on Cyclin-overexpressing T-47D Cells—As a basis for a more detailed examination of the mechanisms responsible for the progestin resistance documented in Figs. 1 and 2, responses in the first 4 days of treatment were characterized. Measurement of cell number over this timeframe revealed a modest decrease in relative cell number after 2 days of treatment of vector-transfected cells (Fig. 3*A*). The initial continuation of cell division in the presence of ORG 2058 is consistent with the early G_1 site of progestin action and consequent ability of cells past this point in the cell cycle to complete a round of DNA replication and mitosis (26). Between days 2 and 4, no further increase in cell number was seen following treatment with either 0.2 or 10 nM ORG 2058 (Fig. 3*A*). Growth curves of cyclin E-overexpressing cells were similar to control cell lines, with an increase in cell number over the first 2 days of treatment but no subsequent increase in the presence of 0.2 or 10 nM ORG 2058 (not shown). In contrast, while the response of cyclin D1-overexpressing cells after 2 days was very similar to that of vector-transfected cells (Fig. 3*B*), thereafter these cells continued to proliferate rather than becoming growth-arrested (Fig. 3*B*).

The relative numbers of vector-transfected cyclin D1 17–1 and cyclin E 17–3 cells after 96 h of treatment with 0.1–10 nM ORG 2058 indicated decreased proliferation following treatment with 0.2 or 10 nM ORG 2058 (Fig. 3*C*). The cyclin D1-overexpressing cell line was relatively insensitive, and the cyclin E-overexpressing cell line was of intermediate sensitivity, consistent with the longer term data presented in Fig. 1. Similarly, measurement of S phase fraction after 48 h of ORG 2058 treatment yielded data (Fig. 3*D*) that paralleled those from the colony-forming assay: the S phase fraction of vector-transfected cells decreased to <20% of control, while the response was substantially attenuated in cyclin D1-overexpressing cells. The two cyclin E-overexpressing cell lines again were inhibited, although to a lesser degree than control cell lines.

After treatment with maximally effective concentrations of ORG 2058 (0.2 or 10 nM) the S phase fraction of vector-transfected control cells decreased from 15–18% to <5% after treatment for ≥30 h (Fig. 4*A*), consistent with previous data (6, 26). The cyclin D1-overexpressing cell line, cyclin D1 17–1, had a higher initial S phase fraction (~22%), although this did not result in a significant increase in proliferation rate (Fig. 3*B*), consistent with other studies of cyclin D1 overexpression (27–29). The S phase fraction was transiently reduced to 11–12% at 18–30 h but maintained at ~18% thereafter (Fig. 4*B*), *i.e.* a

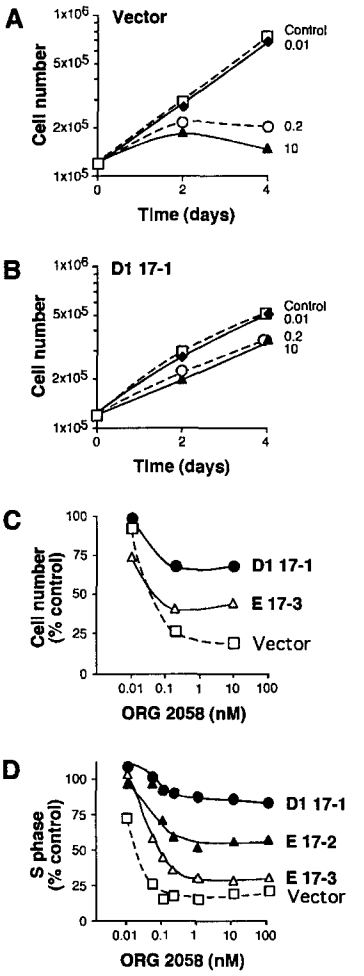

FIG. 3. **Acute progestin inhibition of proliferation in cells overexpressing cyclin D1 or cyclin E.** T-47D cells constitutively expressing cyclin D1 (D1 17–1) or cyclin E (E 17–2, E 17–3) or vector-transfected control cells were treated with the indicated concentrations of progestin (ORG 2058) or vehicle (Control). *A* and *B*, cells were plated into 25-cm^2 flasks 2 days before treatment (day 0). Cell number was determined by hemacytometer cell counting following 2 or 4 days of treatment with ORG 2058 (0.01, 0.2, or 10 nM) or vehicle (Control). The data presented are the means of triplicate cell counts in a representative experiment. The S.E. was smaller than the symbol used in all cases. *C*, cell number following 4 days of ORG 2058 treatment is presented graphically relative to the number of cells in vehicle-treated cultures for each cell line. *D*, cells were harvested for DNA analysis by flow cytometry after 48 h of ORG 2058 treatment. The S phase fraction is presented graphically relative to the value in vehicle-treated cultures of each cell line. Data are representative of two separate experiments.

value similar to that of untreated vector-transfected cells. This S phase value is consistent with the maintenance of proliferation despite the presence of ORG 2058 apparent in Fig. 3*B*. Both cyclin E-overexpressing cell lines also had an increased S phase fraction of 20–22% during exponential growth, again consistent with previous data (28). This was reduced by >50% by treatment with 10 nM ORG 2058, with 0.2 nM ORG 2058 being slightly less effective (Fig. 4, *C* and *D*).

Maintenance of Cyclin Expression Following Progestin Treatment of Cyclin-overexpressing Cells—Western blots of lysates from progestin-treated cells indicated that cyclin D1 expression declined only slightly in cyclin D1 17–1 cells following treatment with either 0.2 or 10 nM ORG 2058 (Fig. 5*B*), in contrast with the ~60% decrease in cyclin D1 expression in either vector-transfected or cyclin E overexpressing cell lines (Fig. 5, *A*, *C* and *D*). Cyclin E levels decreased by 50% after 24–30 h in the cyclin D1-overexpressing cells but recovered to ~80% of control by 48 h (Fig. 5*B*). In neither cyclin E-overexpressing cell

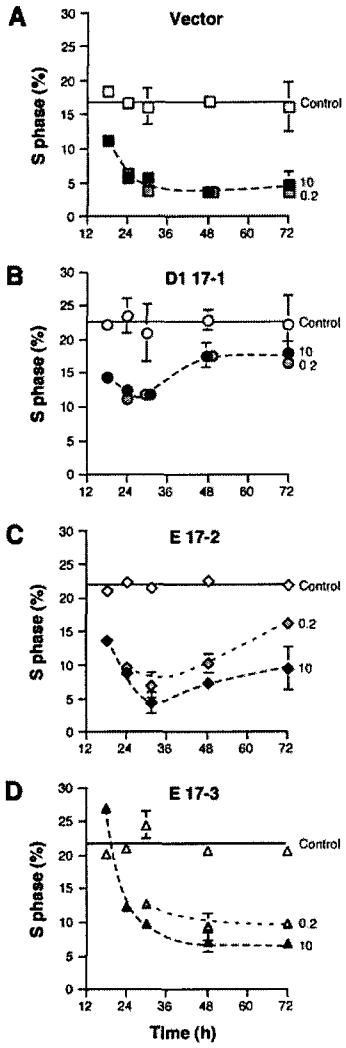

FIG. 4. **Changes in S phase fraction following progestin treatment of cells overexpressing cyclin D1 or E.** T-47D cells constitutively expressing cyclin D1 (D1 17–1) or cyclin E (E 17–2, E 17–3) or vector-transfected control cells were treated with progestin (ORG 2058, 0.2 or 10 nM) or vehicle (Control) and harvested for DNA analysis by flow cytometry at intervals. The S phase fraction is presented graphically as the mean ± S.E. of data from 7 (D1 17–1, E 17–3) or eight (vector, E17–2) separate experiments.

line was cyclin E expression significantly reduced following progestin treatment, but in both cyclin D1 expression decreased to an extent similar to that in control cells (Fig. 5, *A*, *C* and *D*). Thus the level of the exogenously expressed cyclin D1 and E was maintained after progestin treatment.

Both Decreased Cyclin E Expression and p27^{Kip1} Association with Cyclin E-Cdk2 Contribute to the Progestin-mediated Decrease in Cyclin E-Cdk2 Activity—To determine the effects of cyclin E overexpression on cyclin E-Cdk2 activity following progestin treatment, cyclin E-associated kinase activity was measured using an *in vitro* kinase assay (Fig. 6, *A* and *B*). The cyclin E-overexpressing cells displayed a basal level of cyclin E-Cdk2 activity ~2-fold higher than control cells. This was decreased by only ~60% following 10 nM ORG 2058 treatment of cyclin E-overexpressing cells (Fig. 6, *B* and *C*) compared with the ~90% decrease in control cells (Fig. 6, *A* and *C*). Because of the higher initial level of activity, despite this relative decrease the residual cyclin E-Cdk2 activity after progestin treatment was similar to that in untreated control cell lines (compare Figs 6, *A* with *B*). In contrast the S phase fraction of progestin-treated cyclin E 17–3 cells was ~7%, much lower than the 16–17% S phase fraction of untreated control cells (Fig. 4).

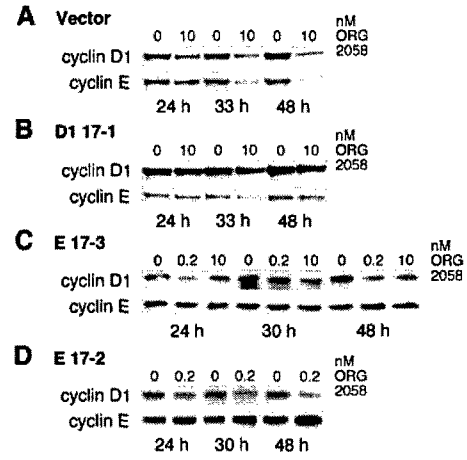

FIG. 5. **Cyclin expression following progestin treatment of cells overexpressing cyclin D1 or E.** T-47D cells constitutively expressing cyclin D1 (D1 17–1) or cyclin E (E 17–2, E 17–3), or vector-transfected control cells were treated with the indicated concentrations of progestin (ORG 2058) or vehicle. Western blots representative of data from three separate experiments for each cell line are shown.

We also examined the phosphorylation of pRb, an endogenous substrate for cyclin E-Cdk2, in lysates of cyclin E-overexpressing cells after ORG 2058 treatment. Hyperphosphorylated, low mobility pRb remained readily detectable following progestin treatment of the cyclin E-overexpressing cell lines but was in low abundance after 32–48 h of ORG 2058 treatment of T-47D tTA-17 cells (Fig. 6, *A* and *B*). Since pRb is phosphorylated by both cyclin E-Cdk2 and cyclin D-Cdk4, progestin effects on Cdk4 activity in cyclin E-overexpressing cells were examined. Cdk4 activity, measured in an *in vitro* assay using pRb substrate, was decreased in both vector-transfected and cyclin E-overexpressing cells to near-baseline levels by 48 h (Fig. 6*D*). Similarly, specific pRb phosphorylation at Ser-780, a site targeted by cyclin D-Cdk4 but not cyclin E-Cdk2 (30), decreased to essentially undetectable levels after 48 h of treatment of either vector-transfected or cyclin E-overexpressing cells (Fig. 6, *A* and *B*). Thus Cdk4 activity was very low following ORG 2058 treatment of either vector-transfected or cyclin E-overexpressing cells, suggesting that the kinase activity responsible for the maintenance of pRb phosphorylation in the cyclin E-overexpressing cells was likely cyclin E-Cdk2. This appears to be sufficient for partial resistance to progestin treatment but not proliferation at control levels.

We previously postulated that the decrease in cyclin E-Cdk2 activity following progestin treatment resulted from both decreased cyclin E expression and increased association of p27^{Kip1} with the remaining cyclin E-Cdk2 complexes (6, 7). To determine the role of p27^{Kip1} association with cyclin E in the decrease in cyclin E-Cdk2 activity in cyclin-overexpressing cells, p27^{Kip1} immunoprecipitates were Western blotted in parallel with the depleted supernatant. In vector-transfected cells few cyclin E-p27^{Kip1} complexes were present in vehicle-treated cells and cyclin E was not significantly depleted by p27^{Kip1} immunoprecipitation (Fig. 7*A*). Cyclin E overexpression increased the number of cyclin E-p27^{Kip1} complexes present in vehicle-treated cells. However, in both cell lines increased cyclin E-p27^{Kip1} association accompanied the decrease in cyclin E-Cdk2 activity following progestin treatment (Fig. 7, *A* and *B*). After ORG 2058 treatment of vector-transfected cells, almost all the cyclin E was associated with p27^{Kip1}, so that little remained in the supernatant after p27^{Kip1} immunoprecipitation (Fig. 7*A*). In contrast, the overexpressed cyclin E was not significantly depleted (Fig. 7*B*). Although cyclin E-p21^{Cip1} complexes were much more abundant in the cyclin E-overexpress-

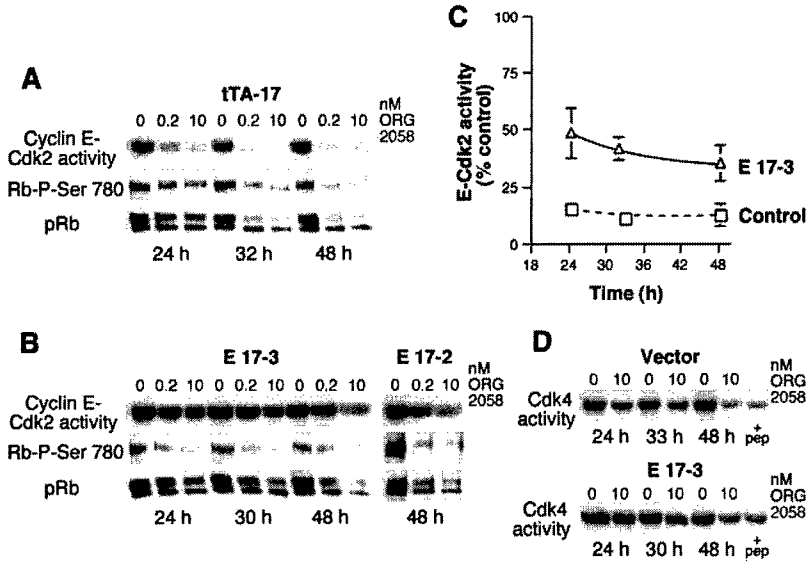

FIG. 6. **CDK activity and pRb phosphorylation following progestin treatment of cells overexpressing cyclin E.** T-47D cells constitutively expressing cyclin E (E 17–2, E 17–3) or parental tTA-17 control cells were treated with the indicated concentrations of progestin (ORG 2058) or vehicle. *A* and *B*, the kinase activity of cyclin E immunoprecipitates from whole cell lysates was measured using histone H1 substrate. Whole cell lysates from the same experiment were also Western blotted for total pRb protein and Cdk4-mediated phosphorylation at Ser-780 (*Rb-P-Ser-780*). *C*, quantitation of data from three separate experiments using 10 nM ORG 2058, with E 17–3 cells presented as mean ± range. Parental, tTA-17, and vector-transfected cells gave similar results and have therefore been pooled (Control). Mean ± S.E. of three to four replicates from four separate experiments are shown. *D*, the kinase activity of Cdk4 immunoprecipitates from whole cell lysates was measured using GST-pRb substrate. The level of background phosphorylation of pRb was estimated by addition of antigenic peptide (+ *pep*) to parallel control samples. Data are from a representative experiment.

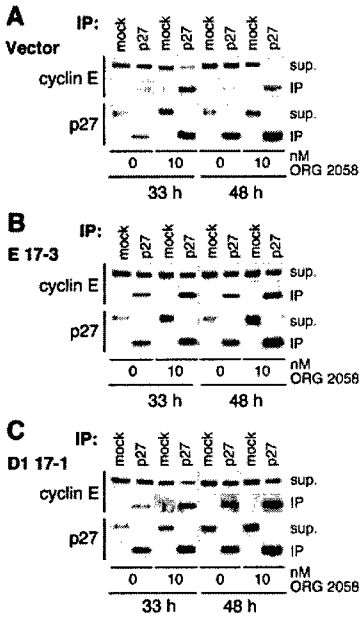

FIG. 7. **Cyclin E-p27^{Kip1} association after progestin treatment of cells overexpressing cyclin D1 or cyclin E.** T-47D cells constitutively expressing cyclin D1 (D1 17–1), cyclin E (E 17–3), or vector-transfected control cells were treated with progestin (ORG 2058, 10 nM) or vehicle. Whole cell lysates were immunoprecipitated by incubation with either p27^{Kip1} antibody followed by protein G-Sepharose beads or protein G-Sepharose beads alone (*mock*). After three rounds of immunoprecipitation, pooled immunoprecipitates (*IP*) and the corresponding supernatants (*sup.*) were Western blotted using the indicated antibodies.

ing cell lines than in control cell lines, they remained in lower abundance than the cyclin E-p27^{Kip1} complexes. Immunoprecipitation of both p27^{Kip1} and p21^{Cip1} did not deplete significant amounts of cyclin E from the cyclin E-overexpressing cells, nor was any change in p21^{Cip1} association with cyclin E observed following progestin treatment (not shown). Thus increased p27^{Kip1} association likely accounts for the 60% decrease in activity following ORG 2058 treatment of cyclin E-overexpressing cells.

Cyclin D1-overexpressing Cells Proliferate in the Presence of ORG 2058 Despite Low Cyclin E-Cdk2 Activity—Measurement of cyclin E-associated kinase activity following progestin treatment of cyclin D1-overexpressing cells revealed an initial decrease of similar magnitude to that observed in control cells *i.e.* to below 25% of that observed in vehicle-treated cells (Fig. 8*A*). However, by 48 h kinase activity had recovered to ~50% of control (Fig. 8*A*), paralleling increased cyclin E abundance at that time point (Fig. 5*B*). Low mobility (hyperphosphorylated) pRb was clearly present following 48 h of progestin treatment of D1 17–1 cells but not vector-transfected cells (Fig. 8*B*), consistent with higher CDK activity in the former cells. Despite the maintenance of cyclin D1 levels following ORG 2058 treatment of cyclin D1-overexpressing cells, specific phosphorylation of pRb at Ser-780 decreased, although it remained at a level significantly above vector-transfected cells treated in parallel (Fig. 8*B*). Measurement of Cdk4 activity using *in vitro* kinase assays confirmed that decreased cyclin D-Cdk4 activity resulted from ORG 2058 treatment of cyclin D1-overexpressing cells but that the decrease in Cdk4 activity was attenuated compared with the >90% decrease in vector-transfected control cells at 48 h (Fig. 8, *B* and *C*). The basal level of kinase activity in these cells was higher than in vector-transfected cells (Fig. 8*B*), such that the residual activity represents ~70% of the level in exponentially proliferating control cell lines.

Recent data showing that lack of p27^{Kip1} can rescue the defects in proliferation resulting from the lack of cyclin D1 (22, 23) argue that sequestration of p27^{Kip1} is important for the physiological role of cyclin D1. However, the similar initial decrease in cyclin E-Cdk2 activity in control and cyclin D1-overexpressing cell lines raised the possibility that p27^{Kip1} redistribution was still occurring in the latter cell line. In vehicle-treated D1 17–1 cells, as in vector-transfected cells, p27^{Kip1} immunoprecipitation did not significantly deplete cyclin E, indicating that little cyclin E was associated with p27^{Kip1} before progestin treatment. ORG 2058 treatment of

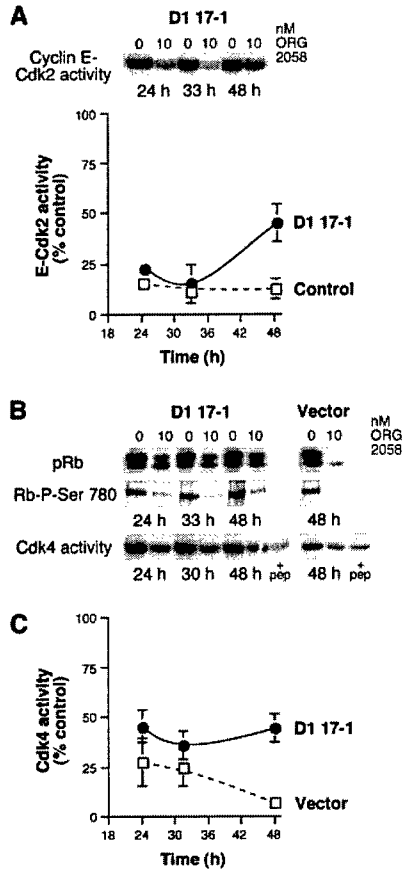

FIG. 8. **CDK activity, pRb phosphorylation, and cyclin E-p27^{Kip1} association following ORG 2058 treatment of cells overexpressing cyclin D1.** T-47D cells constitutively expressing cyclin D1 (D1 17–1) or vector-transfected control cells were treated with progestin (ORG 2058, 10 nM). *A*, the kinase activity of cyclin E immunoprecipitates from whole cell lysates was measured using histone H1 substrate. Quantitation of data from two separate experiments using D1 17–1 cells is presented as mean ± range. Control data presented in Fig. 6 are reproduced for comparison. *B*, whole cell lysates were Western blotted for total pRb protein and phosphorylation at Ser-780 (*Rb-P-Ser 780*), a residue phosphorylated by cyclin D1-Cdk4 and not cyclin E-Cdk2. The kinase activity of Cdk4 immunoprecipitates from whole cell lysates was measured using GST-pRb substrate, with the background pRb phosphorylation estimated by the addition of antigenic peptide (+ *pep*) to parallel control immunoprecipitates. *C*, quantitation of data from two separate experiments with each cell line, presented as mean ± range.

cyclin D1-overexpressing cells increased p27^{Kip1}-cyclin E association at both 33 h, when cyclin E-Cdk2 activity was at its minimum, and 48 h, when it had partially recovered (Fig. 7*C*). At 33 h, the majority of cyclin E was depleted by p27^{Kip1} immunoprecipitation but at 48 h a minority was bound to p27^{Kip1}. Immunoprecipitation of both p27^{Kip1} and p21^{Cip1} confirmed that little cyclin E was bound to p21^{Cip1} after ORG 2058 treatment. Thus much of the cyclin E was not bound to either inhibitor, consistent with the significant cyclin E-Cdk2 activity present at that time point. The increased availability of p27^{Kip1} for cyclin E binding after ORG 2058 treatment was likely due to decreased cyclin D3-Cdk4 complex abundance since there was no detectable decrease in p27^{Kip1}-cyclin D1 association (not shown). Overall these data indicated that cyclin D1 overexpression resulted in progestin resistance independent of effects on p27^{Kip1} availability to bind cyclin E-Cdk2.

DISCUSSION

This study has used T-47D breast cancer cells overexpressing cyclin D1 or cyclin E as a means of investigating the role of these cyclins in progestin inhibition of proliferation. Overexpression of cyclin D1 induced substantial progestin resistance, but cyclin E overexpression did so weakly. This was apparent both in cell lines stably transfected with each cyclin and following retroviral expression of the cyclins. The greater effectiveness of cyclin D1 in inducing progestin resistance does not appear to simply result from higher relative expression, since cell lines overexpressing either cyclin displayed a similar increase in S phase fraction compared with control cell lines, with the implication that the overexpressed cyclins were at functionally equivalent levels. These data complement our previous demonstration that cyclin D1 induction in progestin-treated cells results in re-initiation of cell cycle progression (6) and indicate that regulation of cyclin D1 is a critical element of progestin inhibition of proliferation in breast cancer cells.

The antiproliferative effects of progestins are often viewed as antiestrogenic, and there are a number of similarities in the molecular mechanisms by which antiestrogens and progestins inhibit breast cancer cell proliferation. Both types of compound decrease cyclin D1 expression, consequently decreasing cyclin D1-Cdk4/6 activity and triggering redistribution of CDK inhibitors which in turn contributes to decreased cyclin E-Cdk2 activity (5, 6, 31, 32). Antiestrogen-mediated arrest of breast cancer cells can be overcome by cyclin D1 induction (33, 34), but constitutive cyclin D1 expression does not lead to long term antiestrogen resistance (35).[2] The differences in response to cyclin D1 overexpression indicate important differences between the mechanisms of action of progestins and antiestrogens, *i.e.* that antiestrogens activate growth-inhibitory pathways that are capable of counteracting the effects of cyclin D1 overexpression, whereas progestins do not.

Our previous examination of possible mechanisms for progestin inhibition of cyclin E-Cdk2 activity showed that after progestin treatment essentially all the cyclin E present was in complexes also containing p27^{Kip1} and therefore inactive (7). These data demonstrate a key role for p27^{Kip1} association in the decrease in activity (6). Since much of the cellular complement of cyclin E in T-47D cells is in inactive complexes (7, 36) and there is a poor relationship between cyclin E levels and cyclin E-Cdk2 activity in a series of breast cancer cell lines (36), the contribution of the concomitant decrease in cyclin E abundance was unclear. The present study demonstrates that decreased cyclin E abundance and p27^{Kip1} association with cyclin E-Cdk2 make similar contributions to decreased cyclin E-Cdk2 activity since maintenance of cyclin E levels attenuated the response by approximately half.

Progestin-treated cyclin E-overexpressing cells maintained cyclin E-Cdk2 activity at levels similar to those in exponentially proliferating control cells. This was, however, not sufficient for S phase entry at the same rate as in untreated control cells, which displayed a much higher S phase fraction. A likely explanation is that the accompanying decrease in cyclin D-Cdk4 activity impaired cell cycle progression, although this is perhaps unexpected given evidence from a number of model systems that cyclin E-Cdk2 activity can compensate for lack of cyclin D1-Cdk4 activity. For example, expression of cyclin E can overcome the G_1 block resulting from expression of the cyclin D-Cdk4/6-specific inhibitor p16^{INK4a} or unphosphorylated pRb (37–40). Furthermore, mice in which the coding sequence of the cyclin D1 gene has been replaced by that of cyclin E do not display the proliferative defects in the retina and mammary gland resulting from lack of cyclin D1, indicating that cyclin E can functionally substitute for cyclin D1 in these tissues (41). However, inhibition of cyclin D1 expression or function by antisense or antibody microinjection, by INK4a family inhibitor expression or by specific chemical inhibitors of Cdk4 (42–45) is sufficient to inhibit cell cycle progression. One

interpretation of these data consistent with our observations is that supra-physiological levels of cyclin E-Cdk2 are necessary to overcome a lack of cyclin D1-Cdk4 activity, *i.e.* that the residual level of cyclin E-Cdk2 activity following progestin treatment is not sufficient to compensate for the reduction in cyclin D-Cdk4 activity.

Cdk4 activity was reduced by progestin treatment of cyclin D1-overexpressing cells in the absence of a significant decrease in cyclin D1 abundance. This response may be a consequence of $p18^{INK4c}$ induction, since we have previously shown a transient induction of *INK4c* mRNA 6–18 h after progestin treatment (7), or may result from decreased cyclin D3 levels (5–7) and consequent decrease in cyclin D3-Cdk4 activity. Despite the decrease in Cdk4 activity, these cells maintained an S phase fraction and proliferation rate only slightly lower than that of untreated, exponentially proliferating control cells. Thus, the residual cyclin D-Cdk4 activity after progestin treatment of cyclin D1-overexpressing cells, representing ~70% of that in exponentially proliferating control cells, was sufficient to allow proliferation to continue in the presence of an ~50% decrease in cyclin E-Cdk2 activity. This contrasts with the failure of cyclin E-Cdk2 activity at a level similar to that in untreated control cells to compensate for a lack of cyclin D-Cdk4 activity in progestin-treated cyclin E-overexpressing cells, pointing to functional differences between the two cyclins despite the ability of cyclin E to substitute for cyclin D1 in some model systems (41).

While the ability of cyclin D1 to activate Cdk4/6 has been well studied as a mechanism for its roles in cell cycle progression and oncogenesis, it has additional functions that may be at least as important. These include CDK-independent interactions with transcription factors including a Myb-like protein (DMP1) (46, 47), STAT3 (48) and the estrogen and androgen receptors (49–51). Recent genetic evidence implicates the ability of cyclin D1 to sequester $p27^{Kip1}$ as an essential physiological function for cyclin D1 and has focused attention on this role. Inactivation of the gene encoding $p27^{Kip1}$ corrects the defects in retinal and mammary development resulting from lack of cyclin D1 (22, 23), and substantially overcomes the delay in cell cycle re-entry after serum starvation of fibroblasts lacking Cdk4 (52). These data suggest that increased $p27^{Kip1}$ availability rather than lack of cyclin D1-Cdk4 activity leads to proliferative defects in the retina and mammary gland of mice lacking cyclin D1. In contrast with the prediction of this conclusion, cyclin D1 overexpression in progestin-treated cells prevented inhibition of proliferation but not the decrease in cyclin E-Cdk2 activity resulting from $p27^{Kip1}$ association with these complexes. Thus, this effect of cyclin D1 is not dependent on the ability of cyclin D1 to sequester $p27^{Kip1}$, providing evidence for a critical function for cyclin D1 other than as a high-capacity sink for CDK inhibitors. The observation that cyclin D1 is necessary for cell cycle progression *in vitro* only in cells with functional pRb (53) points to activation of Cdk4/6 and consequent phosphorylation of pRb as the most likely alternative.

The mechanism by which progestins regulate cyclin D1 and E expression remains unknown, although the correspondence between changes in mRNA and protein levels (6) argues that it is via regulation of mRNA abundance. However, the decreases in expression implicated in inhibition of proliferation do not occur within the first 6–12 h of progestin treatment and thus are not likely to be direct effects of progestin. Identifying the pathway(s) responsible for regulation of these cyclins, particularly cyclin D1, will offer further insight into the mechanisms by which progestins inhibit cell proliferation.

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CONSTITUTIVE OVEREXPRESSION OF CYCLIN D1 BUT NOT CYCLIN E CONFERS ACUTE RESISTANCE TO ANTIESTROGENS IN T-47D BREAST CANCER CELLS[1]

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Running title: Cyclin D1 and cyclin E expression and antiestrogen sensitivity

Keywords: cyclin D1; cyclin E; antiestrogen; breast cancer

FOOTNOTES

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[3]The abbreviations used are: ER, estrogen receptor; PR, progesterone receptor; Cdks, cyclin dependent kinases; pRb, retinoblastoma protein.

ABSTRACT

Cyclin D1 and cyclin E are overexpressed in approximately 45% and 30% of breast cancers, respectively, and adverse associations with patient outcome have been reported. The potential roles of cyclin D1 and cyclin E expression as markers of therapeutic responsiveness to the pure steroidal antiestrogen Faslodex (ICI 182780) were investigated using T-47D breast cancer cell lines constitutively overexpressing cyclin D1 or cyclin E. Measurement of S phase fraction, phosphorylation states of the retinoblastoma protein, pRb, and cyclin E-Cdk2 kinase activity demonstrated that overexpression of cyclin D1 decreased sensitivity to antiestrogen inhibition at 24 and 48 hours. Overexpression of cyclin E produced a less pronounced early cell cycle effect indicating only partial resistance to antiestrogen inhibition in the short-term. In Faslodex-treated cyclin D1-overexpressing cells, sufficient Cdk activity was retained to allow pRb phosphorylation and cell proliferation, despite an increase in the association of p21 and p27 with cyclin D1-Cdk4/6 and cyclin E-Cdk2 complexes. In long term colony-forming assays, antiestrogen treatment inhibited colony growth in cyclin D1- or cyclin E-overexpressing breast cancer cells, but with a ~2-2.5 fold decrease in dose sensitivity. This was associated with a reduction in cyclin D1 levels and a decline in cyclin E-Cdk2 activity in cyclin D1-overexpressing cells, and the maintenance of cyclin E-p27 association in the cyclin E-overexpressing cells. These data confirm that cyclin D1 expression and cyclin E-p27 association play an important role in antiestrogen action and suggest that cyclin D1 or cyclin E overexpression has subtle effects on antiestrogen sensitivity. Further studies to identify the mechanism leading to downregulation of cyclin D1 following long-term antiestrogen treatment and to assess the relationship between antiestrogen sensitivity and expression of cyclin D1, cyclin E or p27 in a clinical setting are required.

INTRODUCTION

Breast cancer is a heterogenous disease with regard to its morphology, invasive behavior, metastatic capacity, hormone receptor expression, responsiveness to treatment and clinical outcome. Both estrogen and progesterone receptors (ER^3, PR) are used routinely in the clinical management of breast cancer as predictors of a patient's response to endocrine therapy and as weak prognostic indicators of a patient's clinical course. Two-thirds of all breast cancers are ER-positive and ER is a molecular target for endocrine therapy. The non-steroidal antiestrogen tamoxifen is the endocrine treatment of choice and is used with or without chemotherapy in the management of all stages of ER-positive breast cancer in both pre- and post-menopausal women. However, only ~50% of patients with ER-positive tumors and ~75% of patients with tumors exhibiting both ER- and PR-positivity will respond to endocrine therapy. Moreover, acquired resistance is a major problem in breast cancer management and thus tamoxifen may only be effective for a limited period.

Cancer is a genetic disease where successive mutations lead to the progressive loss of normal homeostatic mechanisms that control cell proliferation, differentiation and death, giving the cell a selective advantage in its environment and leading to clonal expansion. Normal cell proliferation is under strict regulation. There is a physiological 'restriction point' late in G_1 where the cell integrates signals it receives from the internal and external environment and commits itself to passage from G_1 phase to S phase. Beyond this checkpoint, the cell becomes refractory to the effects of external growth stimuli and hormonal influences and is destined to DNA replication and ultimately cell division (1). Estrogen and antiestrogens exert their effects in the G_1 phase of the cell cycle, promoting or inhibiting cell cycle progression. Cyclins belonging to the D and E families and their respective kinase partners, Cdk4/6 and

Cdk2, are involved in late G_1 restriction point control (2, 3). Deregulated expression of cyclin D1 or cyclin E renders growth of normal cells less dependent on growth factors and accelerates passage through G_1 phase of the cell cycle (4). Overexpression of either cyclin D1 or cyclin E leads to mammary carcinoma in transgenic animal models, suggesting a role as oncogenes in mammary epithelium (5, 6). Moreover, both cyclin D1 and cyclin E are overexpressed in a substantial proportion of breast cancers, 45% and 30% respectively (7-9) and some studies have indicated that overexpression of either gene is associated with poor prognosis in breast cancer (8, 10), although other studies have failed to demonstrate such relationships (11-14).

There is now general consensus that cyclin D1 abundance is positively correlated with ER-positivity in breast cancer (13-15). Our earlier studies indicated that both *CCND1* amplification and cyclin D1 mRNA overexpression are associated with poor prognosis in ER-positive breast cancer patients (10, 16). One mechanism by which overexpression of cyclin D1 may lead to a worse clinical outcome is by conferring resistance to endocrine treatment. Consistent with this possibility, a recent clinical study from this laboratory suggested that the duration of the response to tamoxifen was significantly longer in ER-positive patients with low cyclin D1 mRNA levels than in those with high cyclin D1 (10), although these analyses must be interpreted with caution because of the small sample size. Further indirect support for this hypothesis comes from previous *in vitro* studies demonstrating that a reduction in cyclin D1 mRNA and protein expression is an early and critical event in antiestrogen action (17, 18). Another study demonstrated that short-term ectopic induction of cyclin D1 expression in ER-positive breast cancer cell lines (T-47D and MCF-7) can overcome the inhibition of cell cycle progression induced by antiestrogen (19). Together these data suggested that overexpression of cyclin D1 in ER-positive tumors may lead to insensitivity to antiestrogens. However, a more recently

published study indicated that inducible cyclin D1 overexpression in MCF-7 breast cancer cells does not prevent inhibition of cell growth by antiestrogens (20).

Patients with breast cancer overexpressing cyclin E have a significantly increased risk of relapse and death (8, 21). About 40% of breast cancers overexpressing cyclin E have mutant pRb and high $p16^{INK4a}$ expression suggesting that abnormal cyclin E expression may be linked to deregulation of the cyclin D1-Cdk4-$p16^{INK4a}$-pRb pathway (22). Given that cyclin E can functionally replace cyclin D1 in mice (23), overexpression of cyclin E may have effects similar to overexpression of cyclin D1. In contrast to cyclin D1, overexpression of cyclin E is associated with ER-negativity in breast cancer. Although ER-negativity is a good predictor of insensitivity to endocrine treatment in breast cancer, the role of cyclin E as a marker of therapeutic responsiveness to antiestrogens has not been elucidated.

Given that published data provide preliminary evidence that levels of cyclin D1 and cyclin E expression may influence therapeutic sensitivity to antiestrogens, we investigated this hypothesis *in vitro* using clonal T-47D breast cancer cell lines constitutively overexpressing either cyclin D1 or cyclin E.

MATERIALS AND METHODS

Cell Culture

The cell lines derived from T-47D human breast cancer cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and insulin (10 μg/ml). For experiments investigating the short term effects of ICI 182780 150 cm^2 flasks were seeded with 4 x 10^6 cells. ICI 182780 (7α-[9-(4,4,5,5,5-pentafluropentylsulfinyl)

nonyl]estra-1,3,5,(10)-triene-3,17β-diol, a kind gift from Dr Alan Wakeling, Astra Zeneca Pharmaceuticals, Alderley Park, Cheshire, UK) was dissolved in ethanol to 10^{-2} M. The final concentration of ethanol in the tissue culture medium was less than 0.07% and had no effect on the rate of cell proliferation. At the completion of experiments cells were harvested by brief incubation with trypsin (0.05% w/v)/EDTA (0.02% w/v) as previously described (24) or as described below. Cell cycle phase distribution was determined by analytical DNA flow cytometry as described previously (25).

Development of clonal cell lines

The T-47D line from the E. G. and G. Mason Research institute (Worcester, MA) was cloned by limiting dilution, and one clonal cell line, T-47D (7-2), was selected for transfection studies (26). T-47D (7-2) retained the characteristics of the parent line by all tested criteria, in particular sensitivity to growth regulation by steroids and steroid antagonists and abundance of cyclin D1 mRNA. A clonal cell line, Clone 17, was established by transfection of T-47D (7-2) breast cancer cells with the tet-responsive transcriptional activator containing the wild-type Tet repressor and the VP16 activation domain of herpes simplex virus. Further clonal cell lines were established by transfection of Clone 17 cells with empty tetracycline-repressed pTRE vector or full-length cyclin D1 or cyclin E in the pTRE (tet-responsive element) vector (Clontech Laboratories, Palo Alto, USA). Electroporation was carried out in a Bio-Rad Gene Pulser at 950 μF and 0.22 kV/cm. pTK-Hyg was co-transfected into the cells with each gene construct of interest providing a selectable marker. Twenty stable clones transfected with the cyclin D1 construct, 34 clones transfected with the full-length cyclin E construct and three clones transfected with the empty pTRE vector were isolated.

Immunoblot Analysis

Cells were lysed as follows: T-47D cell monolayers were washed twice in ice-cold PBS then scraped into ice-cold lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% [v/v] glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 200 μM sodium orthovanadate, 10 mM pyrophosphate, 100 mM NaF and 1 mM DTT). The lysates were incubated for 5 min on ice and the cellular debris cleared by centrifugation (15,000 x *g*, 5 min, 4°C). Equal amounts of total protein (20-40 μg) were separated by SDS-PAGE then transferred to nitrocellulose filters. Proteins were visualised using the ECL detection system (Amersham, Australia) after incubation (2 h at room temperature or overnight at 4°C) with the following primary antibodies: cyclin D1 (DCS-6) from Novacastra Laboratories Ltd, Newcastle-upon-Tyne, UK; cyclin E (C-19) from Santa Cruz Biotechnology Inc., Santa Cruz, CA; pRb (G3-245) from PharMingen, San Diego, CA; p21 (Cat.#C24420, Transduction Laboratories, Lexington, KY); or p27 (Cat.#K25020, Transduction Laboratories).

Kinase Assay

For assessment of cyclin E-associated kinase activity, cell monolayers were washed twice with PBS then scraped into 1 ml of ice-cold lysis buffer. The lysate was vortexed and placed on ice for 5 - 10 min, then centrifuged at 15,000 x *g* for 5 min at 4°C and the supernatant stored at -80°C. Cyclin E complexes were immunoprecipitated from equivalent amounts of protein with rabbit polyclonal anti-human cyclin E antiserum conjugated to protein A-Sepharose for 2 h at 4°C

(Cat.#14936E, PharMingen). The immunoprecipitates were washed twice with ice-cold 50 mM HEPES pH 7.5, 1 mM DTT.

The kinase reactions were initiated by resuspending the beads in 30 µl kinase buffer (50 mM HEPES, pH 7.5, 1 mM DTT, 2.5 mM EGTA, 10 mM $MgCl_2$, 20 mM ATP, 10 µCi [γ-^{32}P]ATP, 0.1 mM orthovanadate, 1 mM NaF, 10 mM β-glycerophosphate) containing 10 µg histone H1 as a substrate. After incubation for 15 min at 30°C the reactions were terminated by the addition of 10 µl of 3 x SDS sample buffer (187 mM Tris-HCl, pH 6.8, 30% [v/v] glycerol, 6% SDS, 15% [v/v] β-mercaptoethanol). The samples were then incubated at 95°C for 2 min, separated using 10% SDS-PAGE, and the dried gel exposed to X-ray film. Relative band intensities were quantitated by densitometric analysis (Molecular Dynamics, Sunnyvale, CA). Quantitation of protein levels by this method was linear over the range of protein concentrations and exposure times employed in these studies.

Detection of p21- and p27-Associated Proteins

Immunoprecipitation of p21 and p27 was performed using the method described above (for immunoprecipitating cyclin E for kinase activity assays), except that the antibodies were chemically cross-linked to protein A-Sepharose to reduce background (27). Antibodies used were rabbit polyclonal antibodies to human p21 (Santa Cruz Biotechnology Inc., C-19) and human p27 (Santa Cruz Biotechnology Inc., C-19).

The immunoprecipitated proteins were resuspended in 1 x SDS sample buffer, separated by SDS-PAGE, transferred to nitrocellulose membrane and the proteins detected using the antibodies described for Western blotting above.

Colony-forming Assay

Cell viability following drug treatment was assessed in a colony-forming assay. Following harvest from the monolayer, cells were counted, and the appropriate dilutions were made with medium containing the supplements listed above and 5% FCS. The desired number of cells (normally 5×10^3) were plated into duplicate 6-cm plates in 6 ml of medium. The dishes were placed in 37°C incubators with 95% air-5% CO_2 for 21 days.

Following incubation, the medium was removed, and the cells were fixed and stained using the DIFF-Quik STAIN SET 64851 (Lab Aids Pty. Ltd., Narrabeen, Australia). The number of macroscopic colonies were counted using Quantity One 4.2.1 (Bio-Rad Laboratories, Hercules, CA).

RESULTS

Cyclin D1 and cyclin E overexpression in transfected cell lines was maintained during 72 hours of antiestrogen treatment.

In order to address whether cyclin D1 and cyclin E are predictive markers for therapeutic responsiveness to antiestrogens in breast cancer, cell lines overexpressing cyclin D1 or cyclin E were produced using a tetracycline-controlled gene expression system. Two clones overexpressed cyclin E and two overexpressed cyclin D1 in the absence of tetracycline. Although cyclin E expression in clone E 17-3 could be repressed by low concentration of tetracycline (2 μg/ml), the expression of cyclin E in the other clone E 17-2 could not be repressed at all by tetracycline. Repression of

cyclin D1 expression was only achievable with high concentrations of tetracycline (10 – 15 μg/ml) in the two clones overexpressed cyclin D1. In order to avoid the cytotoxic effect of tetracycline, the clones transfected with empty vector were used as control in preference to tetracycline-mediated repression. Western analysis demonstrated that in the absence of tetracycline, clone D1 17-1 constitutively overexpressing cyclin D1 protein by 5-fold, while clones E 17-2 and E 17-3 constitutively overexpressed cyclin E by 6- and 3-fold respectively (Fig. 1A). These T-47D breast cancer cells overexpressed cyclin D1 or cyclin E at levels similar to those previously reported for human breast cancer (7), and were therefore selected for further study.

Inhibition of cyclin D1 gene expression with concurrent decline in cyclin D1 mRNA and protein levels is an early and critical event in antiestrogen action following acute (0 - 48 hours) treatment of T-47D and MCF-7 breast cancer cells with antiestrogens (17, 18, 28). We therefore first tested the effects of the pure steroidal antiestrogen Faslodex (ICI 182780) on the abundance of cyclin D1 or cyclin E protein in the transfected cell lines. In the empty vector cells, cyclin D1 gene expression was downregulated by ICI 182780 (Fig. 1B). In the cyclin D1-overexpressing cell line, cyclin D1 expression was maintained for at least 72 hours following treatment. Cyclin E levels appeared to be slightly reduced by ICI 182780 in the empty vector cells, but increased slightly 24 h following ICI 182780 treatment in both cyclin E overexpressing cell lines (Fig. 1B).

Overexpression of cyclin D1, but not cyclin E, induced antiestrogen resistance in the short term.

S phase fraction was then measured using flow cytometry to assess whether overexpression of cyclin D1 or cyclin E provided any short-term proliferative advantage to cells treated with ICI 182780. Treatment of the cyclin D1-overexpressing cell line led to substantial resistance to antiestrogenic effects on cell cycle progression at 24 hours. The E 17-2 cells demonstrated a much less effect at 24 hours (Fig. 2A). By 48 hour, D1 17-1 became more sensitive to ICI 182780 and by 72 hour, neither cyclin-overexpressing cell line showed any alteration in sensitivity compared to the control cell line (Fig. 2B,C).

Since both cyclin D1 and cyclin E direct the kinase activity of their associated Cdks in phosphorylation of substrates including pRb, the phosphorylation state of the retinoblastoma protein pRb and the Cdk activities are important indicators of cell cycle progression. The hypophosphorylated (faster mobility) form of pRb predominated following 24 h of treatment of the empty vector cells with ICI 182780 in accordance with the inhibition of cell proliferation. The abundance of pRb in the empty vector cells was reduced significantly following 48 hours of antiestrogen treatment. In contrast, hyperphosphorylated pRb remained abundant following treatment of the cyclin D1-overexpressing cell line D1 17-1, consistent with resistance to early cell cycle inhibition. The E 17-2 cell line displayed an intermediate effect, with some reduction in pRb phosphorylation (Fig. 3A). The E 17-3 cell line, expressing a lower level of cyclin E, showed similar effect to the empty cell line, although hyperphosphorylated pRb was still evident at 48 h. These findings are consistent with the results obtained from measurement of S phase fraction (Fig. 2).

The kinase activities of cyclin E-Cdk2 and cyclin D1-Cdk4 were next examined. The abundance of Cdk4-phosphorylated pRb was maintained in the D1 17-1 cells, but reduced in the empty vector and E 17-2 cells after treatment with ICI 182780, indicating that cyclin D1-Cdk4 kinase activity is maintained by overexpression of cyclin D1 (Fig. 3B). Cyclin E-Cdk2 activity decreased in both the empty vector cells and the E 17-2 cells following ICI 182780 treatment. However, the activity was maintained slightly longer in the E 17-2 cells compared with the empty vector cells. The cyclin E-Cdk2 kinase activity was maintained in the D1 17-1 cells for at least 24 hours following ICI 182780 treatment (Fig. 3B), consistent with continued cell proliferation as evidenced from data on pRb phosphorylation and S phase fraction (Fig. 2 and 3A).

Short-term antiestrogen treatment increased p21 and p27 association with cyclin E-Cdk2 complexes in both cyclin D1 and cyclin E overexpressing cells.

A recent study from this laboratory demonstrated that there is a substantial increase in the amount of cyclin E-associated p21 and p27 in MCF-7 breast cancer cells following antiestrogen treatment and the initial decline in cyclin E-Cdk2 activity is dependent on the Cdk inhibitor p21 (28). Thus, the abundance and the distribution of p21 and p27 in each cell line were analysed to determine whether these may be altered by overexpression of cyclin D1 or cyclin E. Total p21 protein peaked 15 h following treatment of the empty vector cells with ICI 182780, an effect similar to that reported in the MCF-7 cells (28). The abundance of both cyclin D1-p21 and cyclin E-p21 complexes was reduced by antiestrogen treatment in empty vector, control cells (Fig. 4A). In contrast, both cyclin D1- and cyclin E-associated p21 was maintained in the cyclin D1-overexpressing cells (Fig. 4B). In the cyclin E-overexpressing cells

following treatment with ICI 182780, although the abundance of cyclin D1-p21 complexes decreased, the abundance of cyclin E-p21 complexes increased (Fig. 4C).

Both p21 and p27 are critical mediators of the therapeutic effects of antiestrogen treatment (29). While p21 appears to be the initiating factor in inhibition of cyclin E-Cdk2 complexes, complete inhibition of kinase activity requires the co-operation of p27 at later time points (28, 29). p27 levels increased modestly following treatment with ICI 182780 in all cell lines (Fig. 5). Little change in cyclin D1-p27 association was apparent in any of the cell lines (Fig. 5). However, cyclin E-p27 association increased following ICI 182780 treatment in all the cell lines. This was apparent as early as 15 h after treatment of the cyclin E-overexpressing cell line (Fig. 5C). The greater increase in p21 and p27 association with cyclin E-Cdk2 complexes by 48 to 72 h in E 17-2 cells (Fig. 4C, 5C) may account for the more effective inhibition of cell proliferation by the antiestrogen in the E 17-2 cells as compared to the D1 17-1 cells (Fig. 2).

Cells overexpressing cyclin D1 or cyclin E retained sensitivity to long-term antiestrogen treatment.

Long-term effects of antiestrogen on cell growth were investigated in a colony-forming assay where cells were treated with ICI 182780 for 3 weeks. In contrast with the attenuation of the short-term antiestrogen effect on cell proliferation in short-term cultures, there was only a very slight decrease in the final level of growth inhibition produced by ICI 182780 in cyclin D1- or cyclin E-overexpressing cells in the long-term clonogenic assays (Fig. 6). No difference was observed between empty vector and E 17-3 cells in the concentration-dependence of inhibition. However, both E 17-2 and D1 17-1 cell lines were less sensitive to ICI 182780 treatment, requiring a concentration of ICI 182780 ~2 - 2.5 times greater for the same degree of inhibition

(Fig. 6B). In addition, a small number of colonies remained following treatment of the D1 17-1 and E 17-2 cells with high concentrations (1 – 10 nM) of ICI 182780 for 3 weeks (Fig. 6B). We therefore investigated whether these colonies had become resistant to ICI 182780. Growth of the residual colonies resumed in the absence of ICI 182780, confirming the cytostatic nature of antiestrogen treatment. After 3 weeks regrowth, cells were replated at the original plating density and exposed to ICI 182780 for a further 3 weeks. Only a small number of colonies were obtained, similar to the original experiment, indicating sensitivity to ICI 182780 had been maintained. Thus the small number of residual colonies present following 3 weeks treatment of D1 17-1 and E 17-2 cells was not due to acquired resistance to antiestrogen.

Sensitivity to long-term antiestrogen treatment was associated with downregulation of cyclin D1 expression in the cyclin D1 overexpressing cells and maintenance of cyclin E-p27 association in the cyclin E overexpressing cells.

The D1 17-1 cells and to a much lesser extent E 17-2 cells were resistant to ICI 182780 in the short-term, but both cell lines became sensitive to the drug in the long term. Western blots of lysates from cyclin D1-overexpressing cells treated with ICI 182780 for 7 or 10 days indicated that cyclin D1 protein levels were reduced by long term antiestrogen treatment (Fig. 7A), and there was also an accompanying reduction in cyclin E-Cdk2 kinase activity (Fig. 7B). Although cyclin E levels were largely unchanged 7 to 10 days following ICI 182780 treatment of the cyclin E-overexpressing cells, a substantial increase in the cyclin E-p27 association was present at these late time points (Fig. 7C), suggesting that this association played an important role in the inhibition of cell growth following the long-term treatment with antiestrogen.

DISCUSSION

Endocrine therapy is an important modality of treatment in all stages of ER-positive breast cancer and tamoxifen has been the therapy of choice for many years. Tamoxifen has demonstrated efficacy in reducing disease recurrence, mortality rate, contralateral breast cancer and in the prevention of breast cancer in high-risk women (30, 31). However, not all ER-positive breast cancers respond to tamoxifen and nearly all patients whose tumors initially respond will develop cellular resistance while maintaining ER-positivity (32). Given that cyclin D1 and cyclin E are overexpressed in a substantial proportion of breast cancer (7, 8) and G_1 cyclins are downstream targets of estrogen-induced mitogenesis (33-35), the cyclins are potential markers of therapeutic responsiveness to antiestrogen. The findings in this *in vitro* study suggest that constitutive overexpression of cyclin D1 and cyclin E interfered with the early cell cycle effects of antiestrogen inhibition. Overexpression of cyclin D1, and to a much lesser extent cyclin E, was associated with increased S phase entry, Cdk activity and pRb phosphorylation following antiestrogen treatment. This is consistent with the previous finding that short-term expression of cyclin D1 under the control of a zinc-inducible metallothionein promoter in ER-positive breast cancer cell lines (T-47D and MCF-7) can overcome the inhibition of cell cycle progression mediated by antiestrogens (19, 25). Despite their increased short-term resistance, T-47D cells constitutively overexpressing cyclin D1 or cyclin E became sensitive to the long-term effects of antiestrogen treatment. This phenomenon was previously observed in MCF-7 cells overexpressing cyclin D1, but the underlying mechanisms responsible for the discrepancy between the short- and long-term effects of antiestrogen were not identified (20).

Estrogens and antiestrogens interact with ER, thereby regulating the transcription of genes that control key points in G_1 progression (18, 28, 34, 35). Antiestrogen treatment leads to a decrease in both cyclin D1 mRNA and protein levels, inactivation of both cyclin D1-Cdk4 and cyclin E-Cdk2 complexes and decreased pRb phosphorylation (18, 28, 36). Previous studies of MCF-7 cells treated with ICI 182780 suggested a model of antiestrogen action in which decreased cyclin D1 abundance is an early and critical event, leading to decreased cyclin D1-Cdk4 activity and increased availability of p21 for cyclin E-Cdk2 binding (18, 28). p21 and p27 association with cyclin E-Cdk2 results in sustained inhibition of this kinase (28, 29). Although some minor differences are apparent, data presented here are consistent with the key features of this model, i.e. the essential role of decreased cyclin D1 expression and p21/p27 association with cyclin E-Cdk2. In the cyclin D1-overexpressing cells, the initial failure of antiestrogen treatment to decrease cyclin D1 expression was accompanied by maintenance of cyclin E-Cdk2 activity and pRb phosphorylation, consistent with the antiestrogen resistance of these cells after 24h treatment and emphasising the central role of cyclin D1.

Although the level of cyclin D1 expression in the cyclin D1-overexpressing cell line was unaffected during the first 3 days of treatment, it was significantly reduced after 7 - 10 days treatment, accompanying the longer-term sensitivity of these cells to antiestrogen treatment. The downregulation of the expression of cyclin D1 in a constitutively overexpressing cell line was unexpected and suggests an increase in the degradation of the protein as a likely mechanism, perhaps via ubiquitin-dependent proteolysis (37-39). In some breast cancers, cyclin D1 overexpression is thought to result from aberrations in proteins involved in cyclin D1 degradation rather than increased mRNA abundance (38).

Ectopic overexpression of cyclin E shortens G_1 phase duration in fibroblasts and HeLa cells (4, 40, 41) and diminishes the serum requirement of cells (41, 42). In cells with inactivation of cyclin D1-Cdk4 by overexpression of p16, pRb can still be phosphorylated by overexpression of cyclin E, indicating that tumors can gain a growth advantage by overexpression of cyclin E (43). Moreover, the phenotypic manifestations of cyclin D1 deficiency can be rescued by cyclin E, demonstrating that cyclin E can functionally replace cyclin D1 (23). The increased abundance of cyclin E in the overexpressing cell line E 17-2 led to an increase in the Cdk2 kinase activity in the absence of antiestrogens. After antiestrogen treatment, decreased cyclin E-Cdk2 activity and pRb phosphorylation was accompanied by an increase in the association of p27 with cyclin E-Cdk2, likely resulting from decreased association of these Cdk inhibitors with cyclin D1. The modest antiestrogen resistance of the cyclin E-overexpressing cells in the short-term and the persistent increase in the association of p27 with cyclin E-Cdk2 suggested that the redistribution of Cdk inhibitors is a significant mechanism contributing to the sensitivity of these cells to antiestrogen.

Patients with relapsed ER-positive breast cancer after initial response to tamoxifen are often treated with second and third line endocrine therapy including aromatase inhibitors and progestin. Given that the major source of estrogen in postmenopausal women is the peripheral aromatisation of estrogen and androgen precursors, the enzyme aromatase has become a major molecular target for endocrine treatment (44-46). Synthetic progestins are an effective therapy in breast cancer and have been used as preferred second-line hormonal agent (47, 48) until the recent emergence of more selective aromatase inhibitors (49). In a parallel study we have recently shown that overexpression of cyclin D1 and to a lesser extent cyclin E can confer resistance to both short- and long-term progestin treatment in T-47D breast cancer cells (50). Although resistance to progestin in cyclin D1-overexpressing breast cancers requires

confirmation in the clinical setting, these *in-vitro* data support the findings from the phase III clinical trials indicating superiority of aromastase inhibitors over progestin (49).

Given most tumors treated with antiestrogen or other endocrine therapy are positive for both ER and cyclin D1, it is therefore important to address whether cyclin D1 has any effect on hormonal responsiveness in the clinical setting. Our previous published study showed that high level of cyclin D1 mRNA was a predictor for worse prognosis with increased risk of relapse, local recurrence, metastases and death in ER-positive breast cancer (10). Further subgroup analysis suggested that high cyclin D1 mRNA level was associated with shorter response duration in primary tamoxifen treatment (10). This hypothesis was further supported by the observation that failure to express both cyclin D1 and ER was a poor prognosis in breast cancer treated with tamoxifen (12).

Numerous mechanisms for the eventual failure of tamoxifen treatment in ER-positive breast cancers have been proposed including elevated estrogen levels, increased tumor antiestrogen binding sites, receptor mutations, impaired signal transduction or alteration of estrogen response elements (32, 51, 52). An increase in estrogen levels or sensitivity may in turn induce transcriptional activation of cyclin D1 expression and potentially increase cyclin D1 protein stability. Given that ectopic overexpression of cyclin D1 can overcome the cell cycle arrest of breast cancer cells (25), escape from the antiestrogen-induced downregulation of cyclin D1 may be a potential mechanism leading to endocrine resistance following long-term tamoxifen treatment. Further clinical studies to correlate cyclin D1 expression with sensitivity to antiestrogen may help in determining the molecular basis of hormonal resistance. Although our *in vitro* study failed to show that ectopic overexpression of cyclin D1

had major effects on antiestrogen-induced growth inhibition, further research is warranted to elucidate the usefulness of measurement of cyclin D1 in selecting the most efficacious endocrine therapy and the contribution of cyclin D1 expression to the development of endocrine resistance in ER-positive breast cancer.

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LEGENDS TO FIGURES

Fig. 1. Levels of cyclin D1 and cyclin E expression in the T-47D clonal cell lines. (A) Total cell lysates from clonal cell lines derived from T-47D and stably transfected with empty vector (Empty) or full length human cyclin D1 (D1 17-1) or cyclin E (E 17-2, E 17-3) were separated by SDS-PAGE and Western blotted for cyclin E and cyclin D1. D1 17-1 overexpressed the cyclin D1 protein by 5-fold and the clones E 17-2 and E 17-3 overexpressed the cyclin E protein by 6- and 3-fold respectively. (B) Western analysis of clonal cell lines treated with and without antioestrogen. Exponentially proliferating cells were treated with 100 nM ICI 182780 (+) or ethanol vehicle (-) and whole cell lysates were prepared at the time points indicated. The 3 lanes to the right of the dotted line contained cell lysates from the empty vector cell line acting as control. The cell lysates were immunoblotted with antibodies to cyclin D1 and cyclin E.

Fig. 2. Acute effects of ICI 182780 on cell proliferation in cyclin D1 and cyclin E overexpressing cell lines. Following treatment of proliferating cells with ICI 182780 over the range of concentrations indicated, cells were harvested and stained with ethidium bromide for analysis by flow cytometry. Changes in S phase fraction for empty vector, D1 17-1 and E 17-2 cells treated with ICI 182780 for (A) 24 h, (B) 48 h, (C) 72 h.

Fig. 3. Acute effects of ICI 182780 on pRb abundance, pRb phosphorylation and Cdk-kinase activity. (A) The experimental design is described in Fig. 1. Total cell lysates from Empty, D 17-1, E 17-2 and E 17-3 cells were Western blotted for pRb. The lower band is the hypophosphorylated pRb identified by faster mobility and the upper band is the hyperphosphorylated pRb (ppRb) identifed by slower mobility on

SDS-PAGE (B) Cyclin E was immunoprecipitated and subjected to an *in vitro* kinase assay using histone H1 as substrate to determine the activity of cyclin E-Cdk2. The amount of pRb phosphorylated by cyclin D1-Cdk4 was determined by separating total cell lysates by SDS-PAGE and immunoblotting with a Cdk4-phosphospecific antibody.

Fig. 4. Acute effects of ICI 182780 on the abundance of p21 and its complexes with cyclin D1 and cyclin E. Total p21 was determined by separation of the whole cell lysates on SDS-PAGE, and immunoblotting with a p21 antibody. The amount of p21 complexed to cyclin D1 and cyclin E was established by immunoprecipitating p21 from the total cell lysates. p21 immunoprecipitates were resolved by SDS-PAGE and subsequently immunoblotted with antibodies to cyclin D1 and cyclin E. (A) Empty, (B) D1 17-1 and (C) E 17-2 cells.

Fig. 5. Acute effects of ICI 182780 on the abundance of p27 and its complexes with cyclin D1 and cyclin E. Total p27 was determined by separation of the whole cell lysates on SDS-PAGE, and immunoblotting with a p27 antibody. The amount of p27 complexed to cyclin D1 and cyclin E was established by immunoprecipitating p27 from the total cell lysates. p27 immunoprecipitates were resolved by SDS-PAGE and subsequently immunoblotted with antibodies to cyclin D1 and cyclin E. (A) Empty, (B) D1 17-1 and (C) E 17-2 cells.

Fig. 6. Long-term effects of ICI 182780 treatment on colony-formation in cyclin D1 and cyclin E overexpressing cell lines. (A) Cells were plated at 5×10^3 / 6-cm^2 plate and subsequently treated for 3 weeks with ICI 182780 over the range of concentrations indicated before fixation and staining. (B) The number of colonies was quantitated as described in the Materials and Methods.

Fig. 7. Long-term effects of ICI 182780 on cyclin D1, cyclin E, p21, p27 and pRb abundance, Cdk kinase activity, and p27 association with cyclin E in cyclin D1 and cyclin E overexpressing cell lines. (A) The experimental design is described in Figure 1. D1 17-1 and E 17-2 cells were treated with ICI 182780 for 7 and 10 days. Total cell lysates were harvested, separated by SDS-PAGE and Western blotted for cyclin E, cyclin D1, p27, p21 and pRb. β-actin was used as a loading control. (B) Cyclin E was immunoprecipitated and subjected to an *in vitro* kinase assay using histone H1 as substrate to determine the activity of cyclin E-Cdk2 in D1 17-1 cells following treatment with ICI 182780 for 7 and 10 days. (C) The amount of p27 complexed to cyclin E at 7 and 10 days following treatment with ICI 182780 was established by immunoprecipitating p27 from the total cell lysates of E 17-2 cells. p27 immunoprecipitates were resolved on SDS-PAGE and subsequently immunoblotted with the antibody to cyclin E.

Figure 1

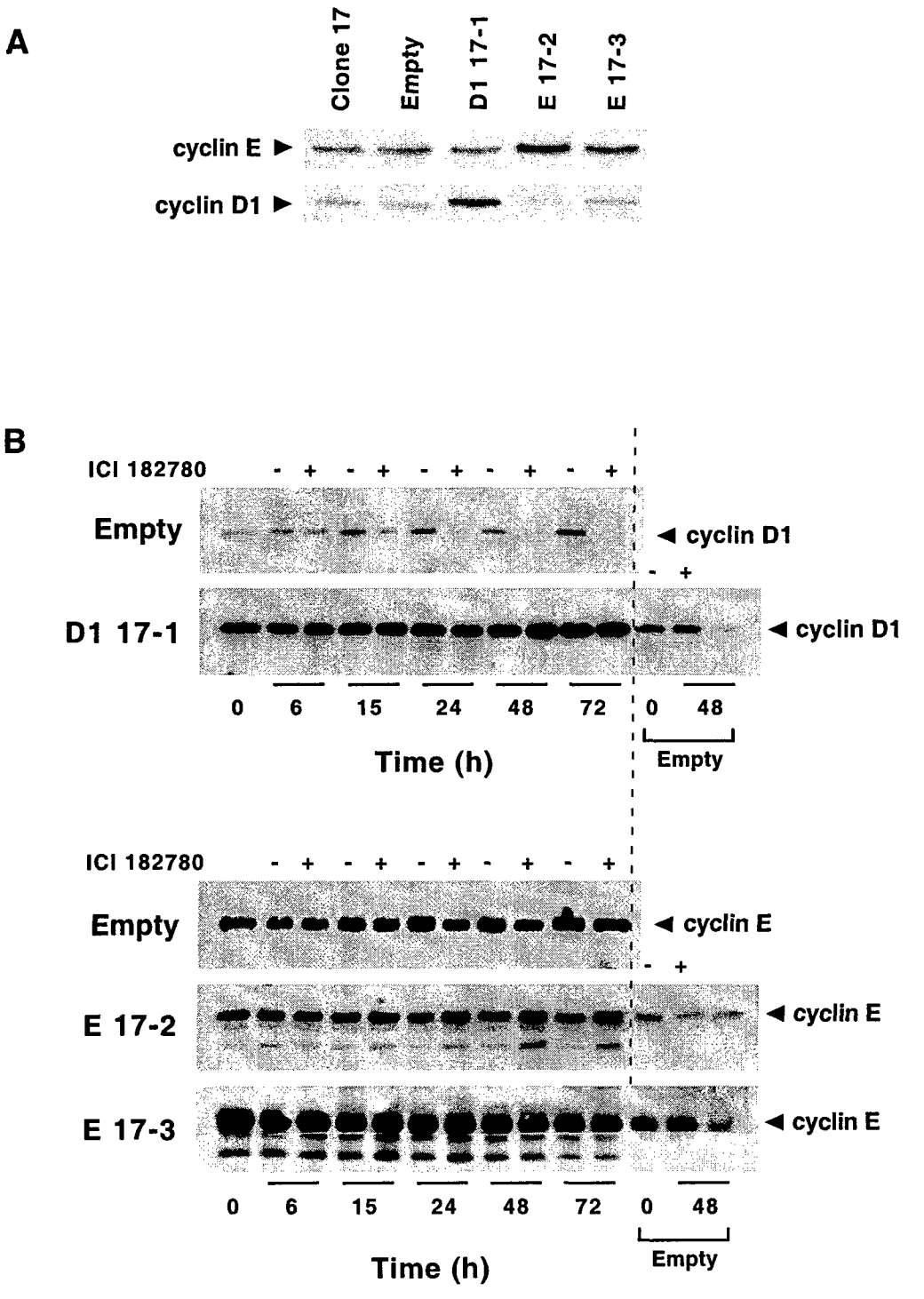

A
Clone 17
Empty
D1 17-1
E 17-2
E 17-3
cyclin E
cyclin D1
B
ICI 182780
- + - + - + - + - +
Empty
cyclin D1
D1 17-1
cyclin D1
0 6 15 24 48 72 0 48
Time (h)
Empty
ICI 182780
- + - + - + - + - +
Empty
cyclin E
E 17-2
cyclin E
E 17-3
cyclin E
0 6 15 24 48 72 0 48
Time (h)
Empty

Figure 2

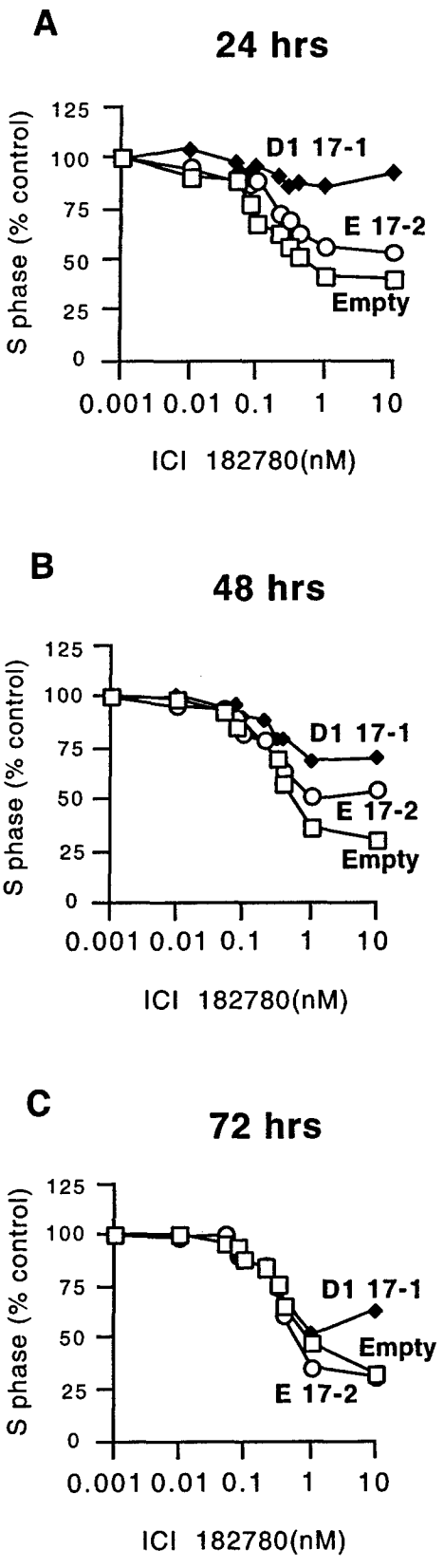
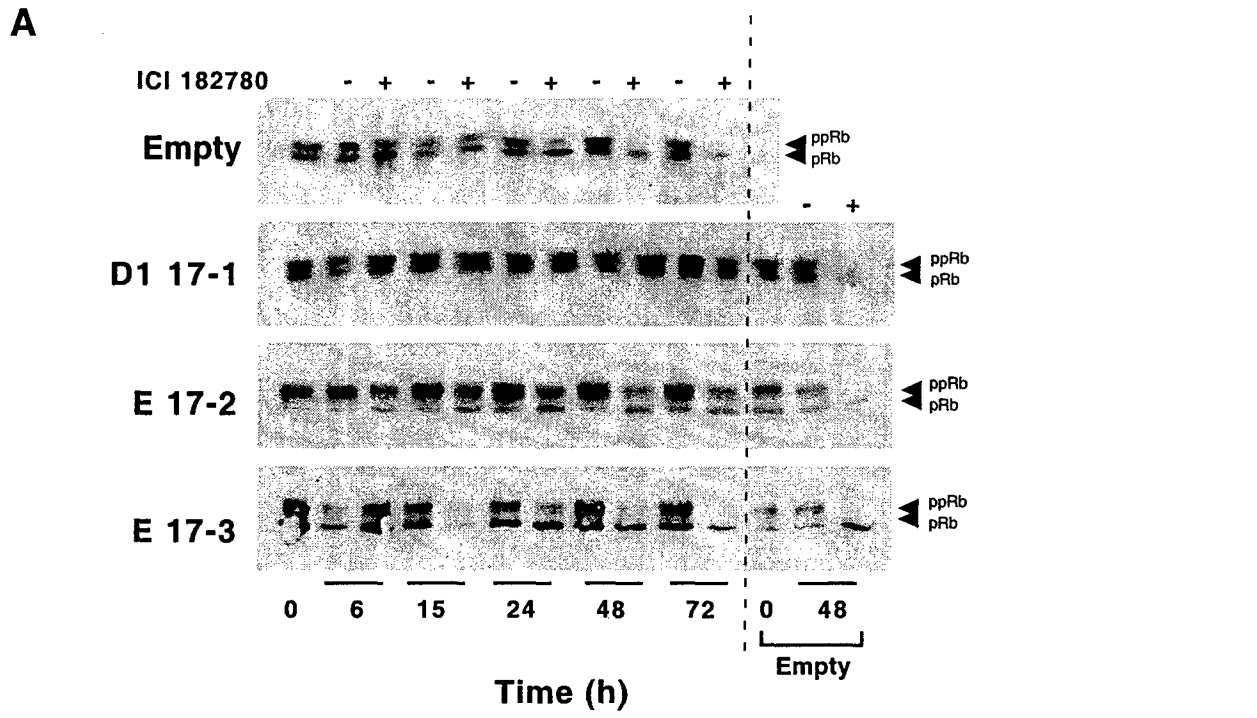

A
24 hrs
S phase (% control)
125
100
75
50
25
0
D1 17-1
E 17-2
Empty
0.001 0.01 0.1 1 10
ICI 182780(nM)
B
48 hrs
S phase (% control)
125
100
75
50
25
0
D1 17-1
E 17-2
Empty
0.001 0.01 0.1 1 10
ICI 182780(nM)
C
72 hrs
S phase (% control)
125
100
75
50
25
0
D1 17-1
Empty
E 17-2
0.001 0.01 0.1 1 10
ICI 182780(nM)

Figure 3


A
ICI 182780
- + - + - + - + - +
Empty
D1 17-1
E 17-2
E 17-3
ppRb
pRb
- +
0 6 15 24 48 72 0 48
Empty
Time (h)

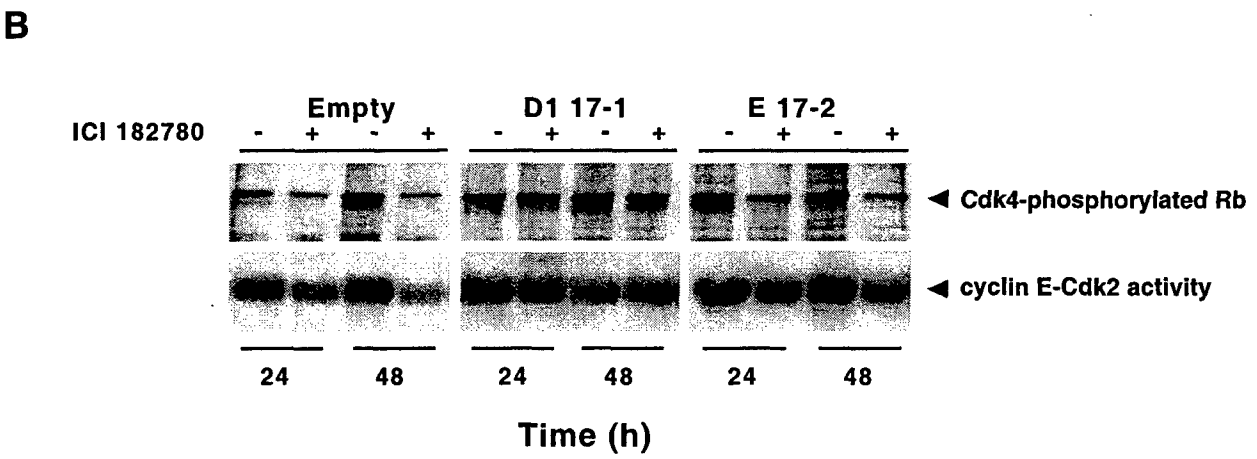

B
Empty
D1 17-1
E 17-2
ICI 182780
- + - + - + - + - + - +
Cdk4-phosphorylated Rb
cyclin E-Cdk2 activity
24 48 24 48 24 48
Time (h)

Figure 4

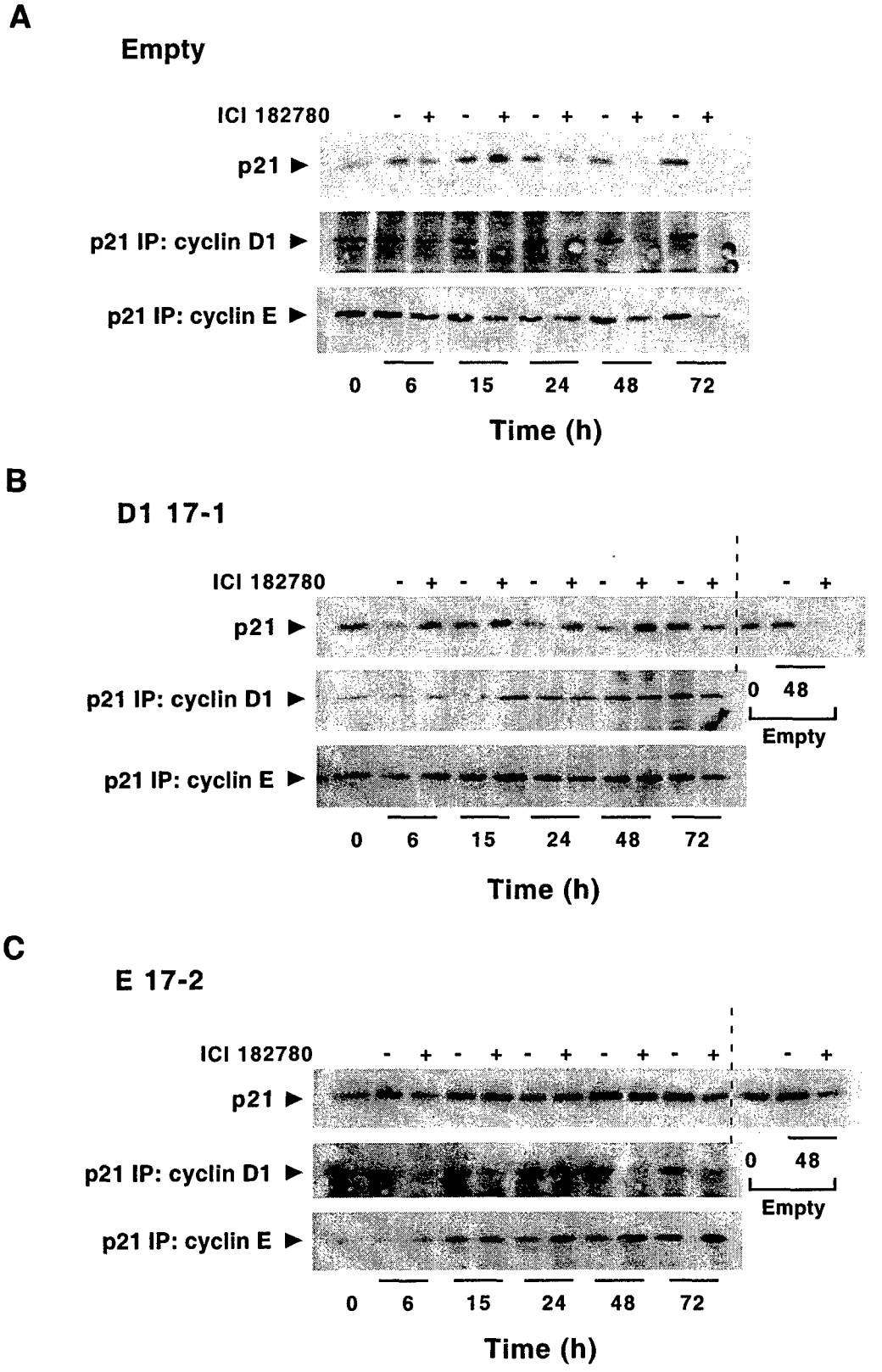

A
Empty
ICI 182780
- + - + - + - + - +
p21 ▶
p21 IP: cyclin D1 ▶
p21 IP: cyclin E ▶
0 6 15 24 48 72
Time (h)
B
D1 17-1
ICI 182780
- + - + - + - + - + - +
p21 ▶
p21 IP: cyclin D1 ▶
p21 IP: cyclin E ▶
0 48
Empty
0 6 15 24 48 72
Time (h)
C
E 17-2
ICI 182780
- + - + - + - + - + - +
p21 ▶
p21 IP: cyclin D1 ▶
p21 IP: cyclin E ▶
0 48
Empty
0 6 15 24 48 72
Time (h)

Figure 5

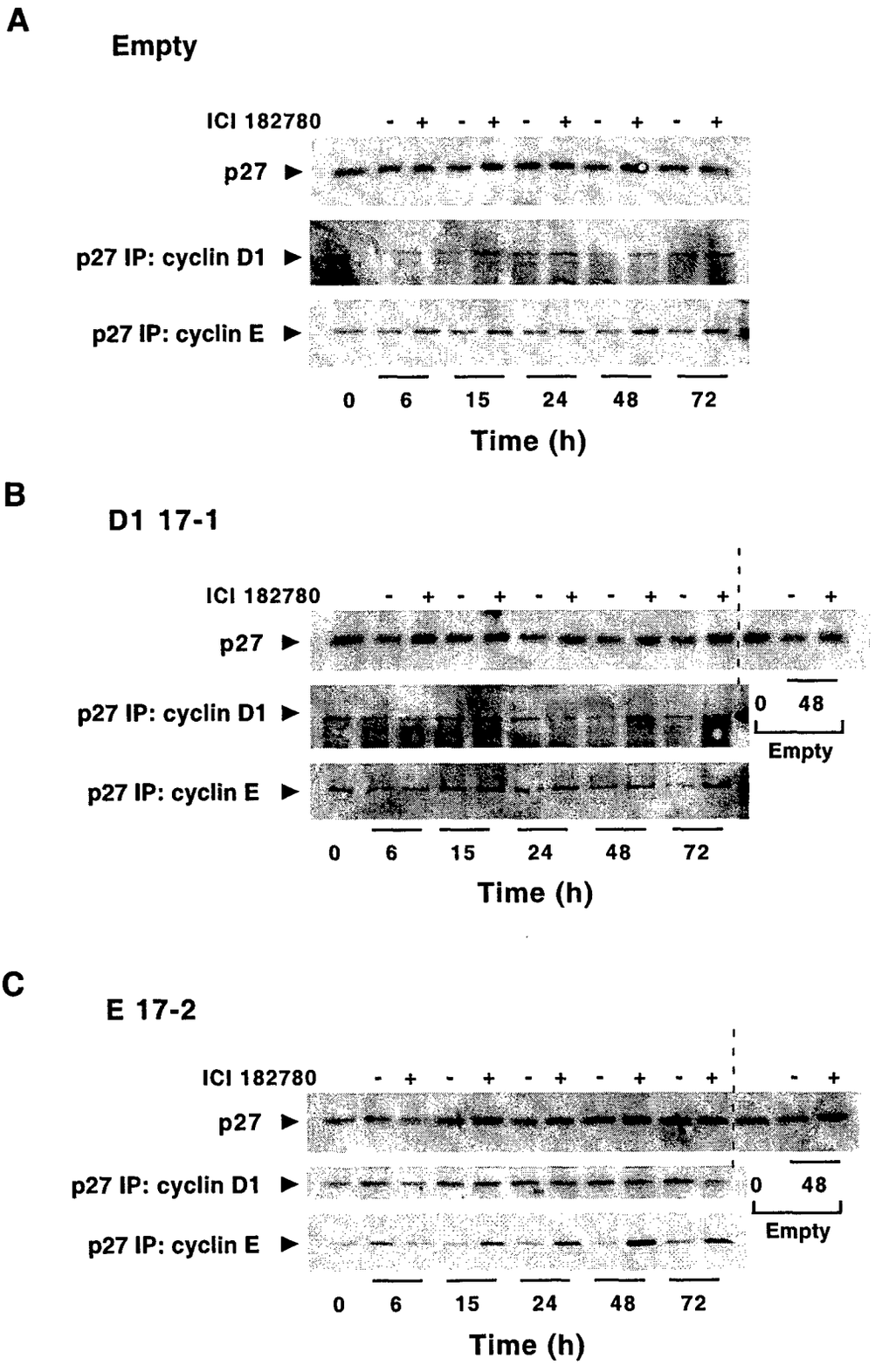
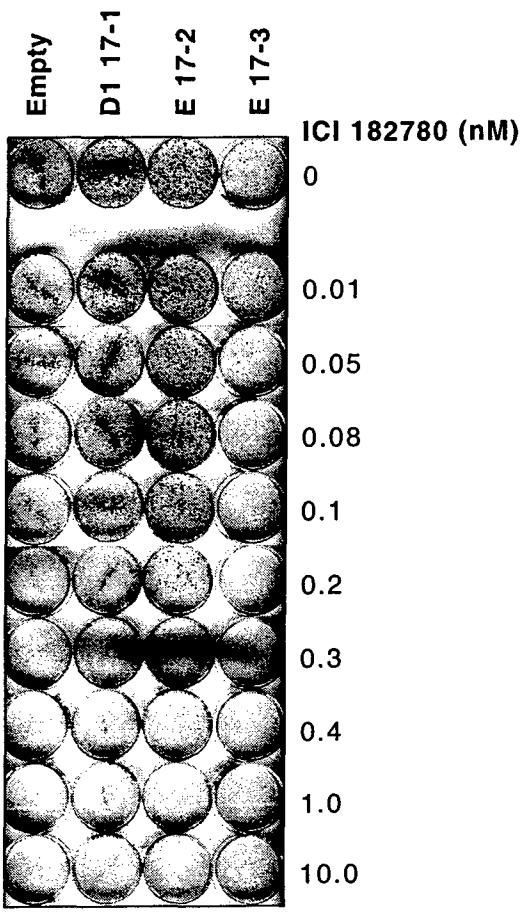

A
Empty
ICI 182780
- + - + - + - + - +
p27
p27 IP: cyclin D1
p27 IP: cyclin E
0 6 15 24 48 72
Time (h)
B
D1 17-1
ICI 182780
- + - + - + - + - + - +
p27
p27 IP: cyclin D1
p27 IP: cyclin E
0 48
Empty
0 6 15 24 48 72
Time (h)
C
E 17-2
ICI 182780
- + - + - + - + - + - +
p27
p27 IP: cyclin D1
p27 IP: cyclin E
0 48
Empty
0 6 15 24 48 72
Time (h)

Figure 6

A


Empty
D1 17-1
E 17-2
E 17-3
ICI 182780 (nM)
0
0.01
0.05
0.08
0.1
0.2
0.3
0.4
1.0
10.0

B

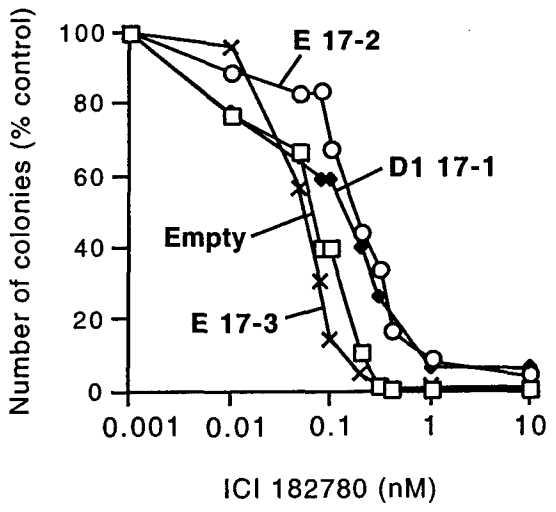
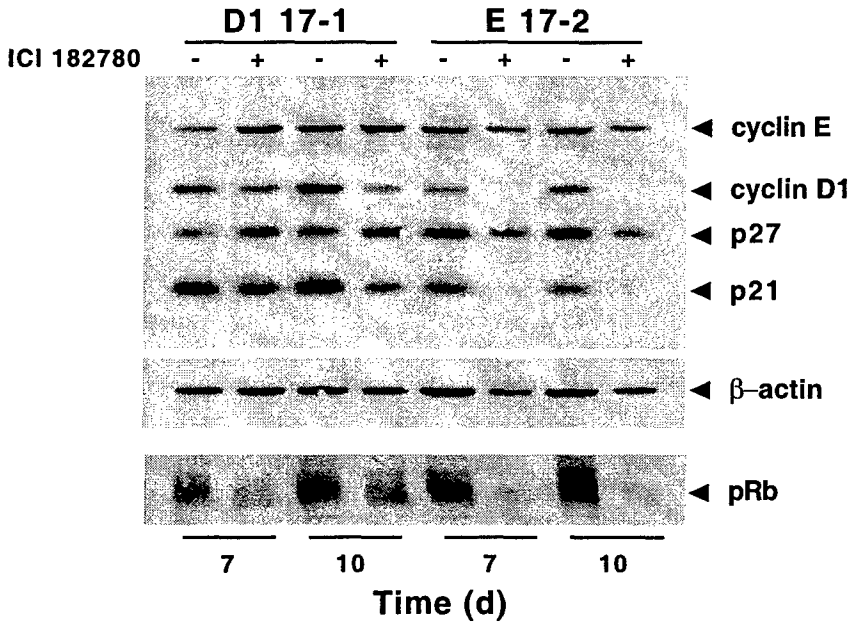
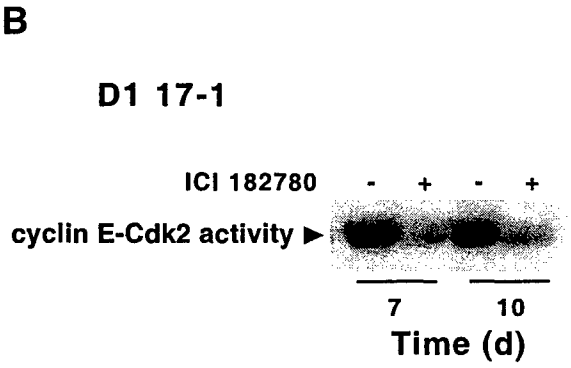

Number of colonies (% control)
100
80
60
40
20
0
E 17-2
D1 17-1
Empty
E 17-3
0.001
0.01
0.1
1
10
ICI 182780 (nM)

Figure 7

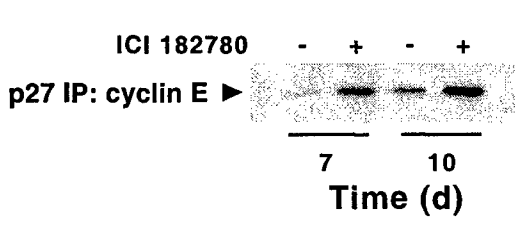
A


D1 17-1
E 17-2
ICI 182780
- + - + - + - +
cyclin E
cyclin D1
p27
p21
β–actin
pRb
7 10 7 10
Time (d)

B


D1 17-1
ICI 182780
- + - +
cyclin E-Cdk2 activity
7 10
Time (d)

C


E 17-2
ICI 182780
- + - +
p27 IP: cyclin E
7 10
Time (d)

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 21 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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